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The effect of Superman, Agamous, and ACS-7 gene manipulations on sex determination in transgenic Fragaria vesca 'Hawaii-4'

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THE EFFECT OF *SUPERMAN*, *AGAMOUS*, AND *ACS-7* GENE MANIPULATIONS ON SEX DETERMINATION IN TRANSGENIC *FRAGARIA VESSCA* 'HAWAII-4'

BY

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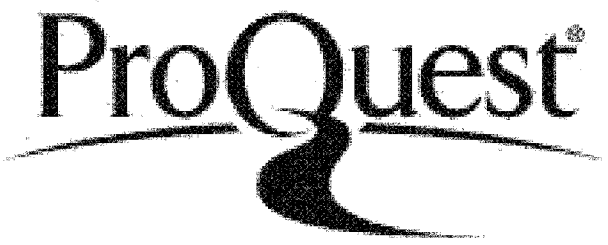

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ABSTRACT

THE EFFECT OF *SUPERMAN*, *AGAMOUS*, AND *ACS-7* GENE MANIPULATIONS ON SEX DETERMINATION IN TRANSGENIC *FRAGARIA VESCA* 'HAWAII-4'

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Xin Ding

University of New Hampshire, May 2012

Sex determination is of interest to plant breeders who transfer useful genes from wild relatives to the cultivated strawberry. The potential roles in sex determination of seven candidate genes, which included five *SUPERMAN* genes, the *AGAMOUS* gene, and the *ACS-7* gene, were studied by re-introducing them into diploid strawberry as overexpression and RNAi constructs by *Agrobacterium*-mediated transformation. All of the transgenic calli expressed the GFP fluorescence marker. Among regenerated plants, some *AGAMOUS*-silenced plants transiently displayed a petalloid stamen or a vestigial petal phenotype, pointing to the *AGAMOUS* gene as negative regulator of A- and B-class genes according to the widely utilized "ABC Model" of flower development. *ACS-7* over-expression and RNAi plants had slower growth rate, which may influence flower development, and the RNAi-transformants did not flower during the study period. Failure to regenerate from callus occurred with over-expression constructs of *FRASUP1, FRASUP3, FRASUP4, FRASUP5*, and *AGAMOUS* and *FRASUP3* RNAi construct.

CHAPTER 1

INTRODUCTION

1.1 Strawberry General Information

Strawberries are among the most popular fruits in the world, and have been utilized by humans for hundreds or even thousands of years (Chinese Pharmacopoeia Commission, 2010). Strawberries belong to the genus *Fragaria* and the Rosaceae family, one of the largest families in the plant kingdom. Other popular rosaceous fruits and ornamentals include: apples (*Malus*); pears (*Pyrus*); cherries, peaches, plums, apricots, almonds (*Prunus*); blackberries and raspberries (*Rubus*); roses (*Rosa*); cinquefoil (*Potentilla*); and hawthorn (*Crataegus*). These plants have a substantial market value, and are of worldwide economic importance. In 2004, world production of strawberries was estimated at 6.9 billion pounds, with the United States contributing 2.2 billion pounds (Rieger, 2004, WS-1). The market value for strawberries produced in the U.S. in 2010 was $2.2 billion (WS-2).

The genus *Fragaria* has intrigued generations of scientists not only because of its economic importance, but because of its inherent genetic interest. As an octoploid (2n=8x=56), the cultivated strawberry *Fragaria* x *ananassa* is among the most genomically complex crop plants (Folta and Davis, 2006). A reportedly small haploid genome size of 206 Mb (Folta and Davis, 2006) promoted the diploid *Fragaria vesca* ($2n = 2x = 14$) as a model for Rosaceae functional genomics (Shulaev et al., 2008). Genetically speaking, *Fragaria* is

appealing for study because it exemplifies the phenomenon of polyploidy (Davis et al., 2007). Unlike animals, plants are tolerant of polyploidy, and in fact polyploidy appears to be an essential part of plant evolution. It is believed that most if not all plant families had one or more polyploidization events in their past (Blanc and Wolfe, 2004), helping to explain why our plant kingdom has become so varied and splendid.

The genus *Fragaria* contains around twenty-one species, ranging in ploidy from diploid to decaploid (Hummer and Hancock, 2009). It not only has a wide range of ploidy levels, but also has a high interspecific hybridization ability which is thought to give rise to speciation, the evidence of which might be found in the genomic organization of the three strawberry octoploid ($2n = 8x = 56$) species *F. ×ananassa*, *F. virginiana* and *F. chiloensis* (Rousseau-Gueutin, 2008). These are reasons, among others, that make *Fragaria* an interesting subject for genetic research.

Among the whole *Fragaria* genus, one species, *Fragaria ×ananassa* Duchesne, is grown commercially from tropical climates to sub-arctic regions. *F. ×ananassa* is the original product of an accidental cross between two wild octoploid species: *F. chiloensis* as female and *F. virginiana* (Darrow, 1966), and has been in cultivation for slightly over 250 years. This relatively young species, well known as the cultivated or dessert strawberry, has large sweet fruits and a distinctive strawberry taste. The two parent species are both indigenous to the Americas. *F. virginiana* is found in eastern portion of North America while *F. chiloensis* is found on the western coasts of both North and South America

(Staudt, 1999, Hummer and Hancock, 2009). Following the colonization of the Americas by Europeans, these two geographically isolated species were independently brought to Europe, where they were grown side by side in novelty gardens. In the early to mid-18th century (Staudt, 1962), pollination by *F. virginiana* of a female clone of *F. chiloensis* serving as the seed parent gave rise to *Fragaria* × *ananassa*. Its melding of the large fruit size of *F. chiloensis* and distinctive sweetness and taste of *F. virginiana* led to the great economic success of this new species.

The genomic makeup of the octoploid species is fascinating. Three models of subgenomic composition have been published on *Fragaria* × *ananassa*. All are based on cytological evidence of chromosome pairing and segregation. The first model contends that the genome of *F.* ×*ananassa* contains three subgenomes, designated AAAABBCC. The three subgenomes represent three different genome contributors in the octoploids' evolutionary past (Fedorova, 1946). The second model states that *F.* ×*ananassa* is composed of three subgenomes, AAA'A'BBBB, but two of those subgenomes, A and A', are more closely related to each other than to the B subgenome (Senanayake and Bringhurst, 1967). The third model presents that *F.* ×*ananassa* has four subgenomes, AAA'A'BBB'B', which are still distinct subgenomes. Subgenomes A and A' are evolutionary closer to each other than to any B subgenome, and vice versa, making *F.* ×*ananassa* fully diploidized (acts like a diploid) (Bringhurst, 1990).

1.2 Importance of Strawberry Sex Determination

By considering the strawberry fruit as the foremost concern, the flower, the sexual organ, is also put under a spotlight, especially for its role in the cross-pollinations used in breeding for varietal improvement. Even though many breeding approaches have been actively pursued, including hybridizations between the cultivated strawberry and the wide diversity of strawberry wild relatives from which desirable traits could be introduced into the cultivated strawberry (Hancock and Luby, 1993), limitations to interspecific hybridization existed, such as differing ploidy levels and mating systems. Logically, *F. virginiana* and *F. chiloensis*, as the direct progenitors, are prime candidates as sources for introducing economically important traits into *F. ×ananassa* via introgression. However, *F. virginiana* and *F. chiloensis*, in the wild, do not have the same mating system as the cultivated *F. ×ananassa*. *F. virginiana* and *F. chiloensis* are typically gynodioecious, and certain populations may exhibit trioecy (Staudt, 2009), whereas, in *F. ×ananassa* hermaphroditism is the general rule, as is preferred by the strawberry production industry. Hermaphroditism, with both male and female sex organs on the same flower, is ideal for large–scale agricultural production as it maximizes the chances of pollination and fruit numbers. Gynodioecious species contain members that are either hermaphroditic or female, whereas trioecious species contain members that are female, male, or hermaphroditic. Neither of the non-hermaphroditic traits is preferred for cultivation due to the reduced chance of pollination and number of fruitable flowers. Also, functionally male plants do not make fruit at all.

The differing sex morphs of *F. virginiana* and *F. chiloensis* present a potential problem for the introgression of economically important traits into *F. ×ananassa*. It is always possible to use wild hermaphrodite relatives for introgression; however this may not always be a satisfactory option, as when a desirable trait in a non-hermaphroditic source is closely linked to a sex determination locus. Therefore it is of practical as well as basic scientific importance to understand the genetic basis of sex determination in *Fragaria*.

1.3 Sex Determination in Octoploids

All known species of polyploid strawberries display some form of sex differentiation, even in the naturally occurring *F. ×ananassa* subspecies *cuneifolia*, which is not cultivated but arises in the Pacific Northwest where the ranges of *F. chiloensis* and *F. virginiana* overlap (Staudt, 2009). Given this fact, sex differentiation might have evolved several times in the genus (Staudt, 2009). Ahmadi and Bringhurst (1991) presented a sex determination model in the octoploid species *F. virginiana* and *F. chiloensis* where femaleness (F), hermaphroditism (H), and maleness (M), have alternate alleles (F, H, M) at a single locus. Under this model, the femaleness allele (F) is dominant to the hermaphroditism allele (H), while the hermaphroditism allele (H) is in turn dominant to the maleness allele (M). Under this model, a hermaphrodite could be either homozygous (H/H) or heterozygous (H/M), while males are always homozygous (M/M). Females are always heterozygous (F/H or F/M). Hence, the segregation ratio female to hermaphrodite (or male) would be always be 1:1 in

the progeny of female x hermaphrodite or female x male, except in the mating F/M x H/M, which yields a 2:1:1 female to hermaphrodite to male ratio.

Spigler et al. (2008, 2010) proposed that in *F. virginiana* the sex determination mechanism does not obey the Ahmadi and Bringhurst (1991) model. Spigler et al. asserted that the male and female determining loci were distinct rather than the same. These authors mapped an *F. virginiana* population of an F1 from a cross between a female and hermaphrodite and found that 2 out of 184 F1 offspring were neuter, lacking both male and female fertility. According to these results, they concluded that male and female fertility were controlled by two closely linked loci with a low frequency of recombination, the two observed neuter types being identified as rare recombinants (Spigler et al. 2008).

1.4 Candidate Genes Approach

Although abundant research has been done in plant flower development, and many related genes have been isolated and characterized since the 1990's (Albani and Coupland, 2010; van Nocker and Ek-Ramos, 2007), few results about sex determination in the genus *Fragaria* have been reported. One logical way to study sex determination in strawberry is to choose the genes known to affect sexual development in those well-studied model species, such as *Arabidopsis*, *Petunia*, and *Antirrhinum*, and then isolate and manipulate their homologues in strawberry genomes.

The method known as the candidate gene approach utilizes the transferability of knowledge of gene function in one organism to the study of an

unknown but possibly related genetic phenomenon in another organism. In this study, a total of seven *Fragaria* candidate genes belonging to three gene families were chosen to see if any of them were involved in sex determination: five members of the *SUPERMAN*-like gene family of putative transcription factors; along with the *AGAMOUS*--containing transcription factor--and the ethylene biosynthesis gene *ACS-7*. Locations of these seven genes on the *Fragaria vesca* linkage map, as determined by Ben Orcheski (2010), are shown in the Figure 1. The reasons why these particular genes were chosen for further study are detailed below.

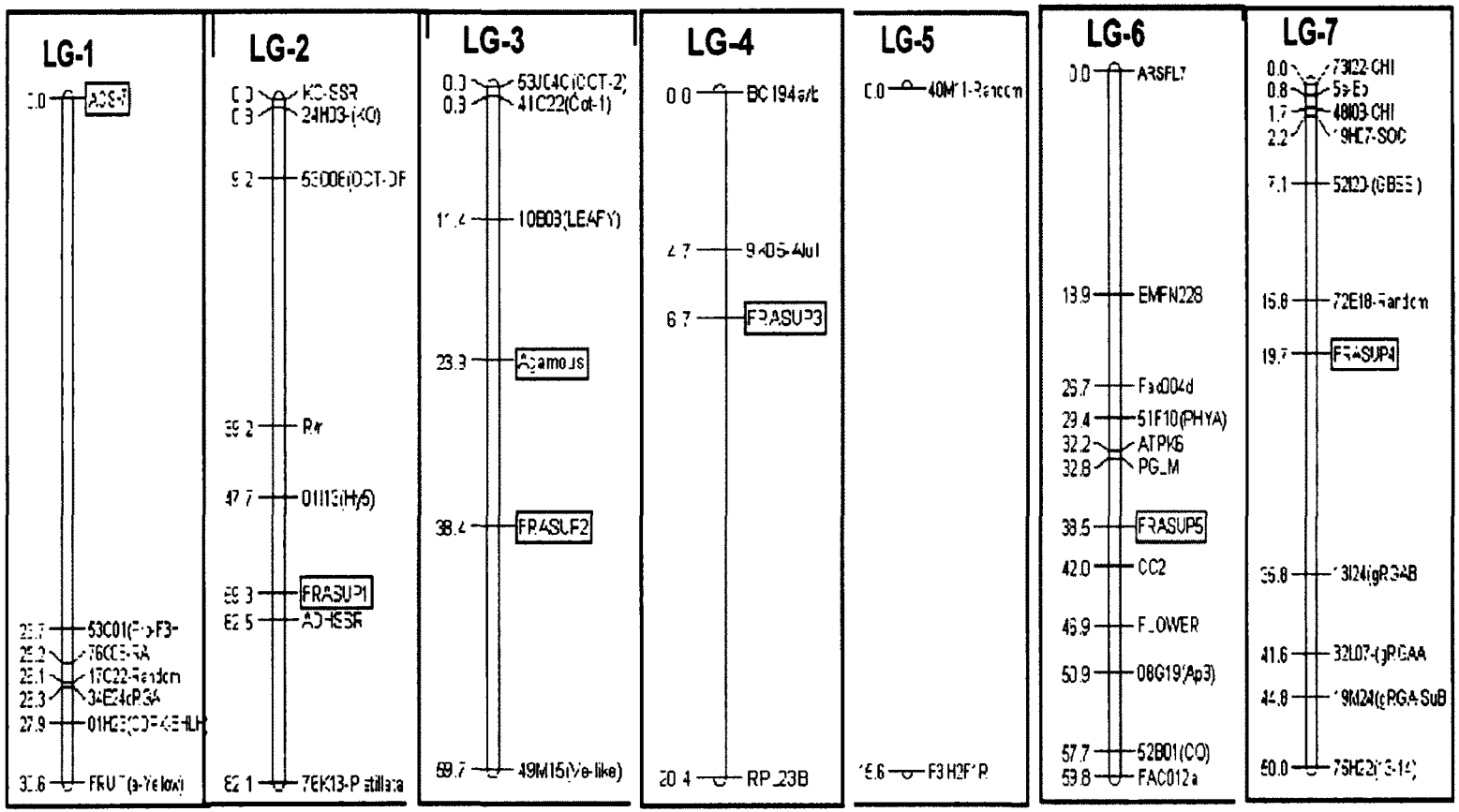


Figure 1. Candidate Genes Location Map. The seven calculated linkage groups derived from the *F. vesca* YP mapping population. The marker name is on the right while the linkage group position (in centimorgans) is on the left. The candidate genes are boxed red. The total combined length of the map is 318.2 cM. From Orcheski (2010).

1.5 The ABC Floral Homeotic Genes

Introduction

The genes that are known to control the sexual organs in the flowers of model species such as *Arabidopsis thaliana* and others require consideration as candidate genes for the study of sex determination in strawberry. The interactions of a set of well-studied genes involved in flower development are described by the genetic model of flower development in *Arabidopsis* (Meyerowitz et al. 1991), as detailed below.

A typical perfect flower is composed of four layers, or "whorls", of metamorphosed leaves. The outer-most layer consists of sepals, which protect the unopened flower. The second layer consists of petals, an attractive organ to pollinators. These two whorls affect plant reproduction indirectly. The third layer consists of stamens, which are the male reproductive structures that produce pollen. In the center of the flower is the fourth whorl, consisting of the pistil or pistils. A pistil consists of one or more carpels, which are female reproductive structures that contain ovules. Stamens and pistils play a direct and important role in plant reproduction. Thus, an initial focus on genes that regulate development of reproductive organs seems logical as a starting place for investigating the genetics of sex determination in *Fragaria*.

Based on genetic mapping done in the late 1980's to early 1990's, we understand that the four whorls of organs in a wild type flower develop under the influence of differential expression patterns of three classes of genes, A, B, and C, as described by the "ABC Model of Flower Development" (Figure 2)

(Meyerowitz et al. 1991; Weigel and Meyerowitz, 1994). These genes were first discovered in the model plant *Arabidopsis thaliana*, and a total of five were characterized: *APETALA1* (*AP1*), *APETALA2* (*AP2*), *APETALA3* (*AP3*), *PISTILLATA* (*PI*), and *AGAMOUS* (*AG*) (Parenicová 2003). Based on this model, *AP1* and *AP2* are A-class genes, *AP3* and *PI* are B-class genes, and *AG* is the lone C-class gene. A-class genes individually direct sepal development, A-class and B-class genes together direct petal development, B-class and C-class genes together direct stamen development, and the C-class gene individually directs carpel development. (Ng 2000)

These five genes are Floral Homeotic Genes (FHG), and are analogous to those homeotic genes directing embryo development in Drosophila: they lead to misplacement of the normal organ if mutated (either by loss of function or overexpression). Accordingly, we assume that ABC homeotic genes, when mutated, would be able to cause a similar phenomenon of organ misplacement in a flower.

The B-class (*AP3* and *PI*) and the C-class (*AG*) genes were initially chosen by Orcheski (2010) as primary candidate genes for sex determination in *Fragaria*, since they direct the growth of the reproductive organs. However, it is important to be aware that FHG classes do not act independently, but instead constitute a complex network. As a result, mutations in FHG genes can lead to various phenotypes.

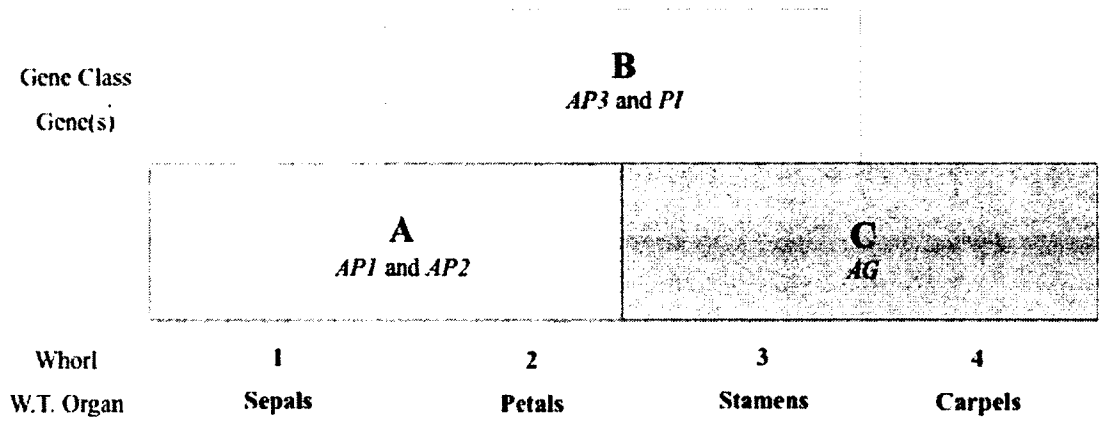


Figure 2. The ABC Model of Flower Development. The wild-type gene expression pattern in a perfect (hermaphroditic) flower based on the "ABC Model of Flower Development" (Meyerowitz et al. 1991). Whorl 1, composed of sepals, requires the expression of the A-class genes, *AP1* and *AP2*. Whorl 2, composed of petals, requires A-class as well as the B-class genes *AP3* and *PI*. Stamens comprise whorl 3 and rely on B-class expression as well as the C-class gene *AG*. The C-class gene *AG* acts alone in the whorl 4 to control carpel development.

The following sections review what is known about genes involved in the development of the plant sex organs: stamens and carpels, with some reference to the classical model of sex determination in *Fragaria*. The B-class genes will be considered first.

APETALA3* and *PISTILLATA

These two B-class genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), mediate petal development in the second whorl and stamen development in the third whorl of wild type flowers. As shown in Figure 2, the two genes, *AP3* and *PI*, work together to induce transcription of genes, and coordinate with A-class and C-class genes to develop petals and stamens, respectively. During the process, *AP3* and *PI* function independently but are required, along with named A-class and C-class genes, to develop organs (Weigel and Meyerowitz, 1994).

In *Arabidopsis*, loss of function of either *AP3* or *PI* causes an inactivity of B-class genes and is characterized by the conversion of second whorl petals to sepals and third whorl stamens to carpels (Bowman et al. 1991; Coen and Meyerowitz, 1991). In some severe functional loss of *PI* alleles, some organ primordia in the third whorl are absent. But whorls one and four do not change since B-class genes do not mediate them (Bowman et al. 1991). Accordingly, we could predict that in some allelic variants of *Fragaria*, malfunction of B-class genes could result in conversion of a perfect to a female flower, that is, the stamens become vestigial and partially convert into carpelloid tissue. However, in normal female strawberry flowers, while we do see the whorl 3 loss of stamens,

we do not see any change in whorl 2 petals, which would be expected if the B-class genes were responsible for this change (Figure 4).

The *APETALA3* gene has elements in the promoter that are specific to either second or third whorl expression (Hill et al., 1998), and maybe it could explain the occurrence of vestigial or carpelloid stamens: the sequences of *AP3* and *PI* have a high degree of similarity, therefore *PISTILLATA* is likely to contain whorl specific promoter elements as well, and those mutant alleles might only have elements that cause the expression in the third whorls but not in the second whorls.

When overexpressed, *APETALA3* and *PISTILLATA* mutants in *Arabidopsis* display drastic deviations from the wild type flower morphology (Krizek and Meyerowitz, 1996; Mizukami and Ma, 1992). Jack et al. (1994) performed an overexpression study and found that when the *AP3* gene was overexpressed under control of a constitutive 35S promoter, carpels converted into stamens. In their study, they generated transgenic 35S::*PI* *Arabidopsis* plants that showed a partial conversion of first whorl sepals to petals. When the two single mutant lines were crossed to create a 35S::*AP3* and 35S::*PI* double mutant, the transgenic plants showed a complete conversion of sepals to petals and carpels to stamens. Thus, in the double mutants, they inferred that the presence of *AP3* enhances the conversion shown in the 35S::*PI* single mutant.. However, neither of the overexpression phenotypes observed in Jack's work matches the characteristics of female flowers in *Fragaria*. Normal female strawberry flowers retain their first whorl sepals and fourth whorl carpels. So, the

female trait in *F. virginiana* is seems unlikely to be the result of overexpression of either of the B-class genes.

With the current understanding of ABC model, the B-class genes would appear to have control over the A- and C-class genes, therefore mutation or manipulation of the B-class genes could lead to development of male flowers through the conversion of carpels into stamens (Jack et al., 1994). However, to conform to the classical model of Fragaria sex determination (Bringhurst, 1991), in which the F, H, and M alleles are all alleles of the same locus, it would need to be possible for different mutant alleles of a B-class gene to determine either femaleness or maleness. But this has not been seen, and Jack's (1994) and Bringhurst's (1991) conclusions fail to explain further work that has been done on sex-determination genes, as explained below.

The single C-class gene is considered next.

AGAMOUS

AGAMOUS (*AG*) is the single C-class floral homeotic gene and was the first FHG to be cloned and sequenced among all the ABC genes. *AGAMOUS* has the distinction not only of being the best characterized of the ABC genes (Yanofsky et al. 1990), but also of playing two key roles in proper flower development:

First, according to the ABC model, *AG* mediates organ development in flower whorls three and four. In the third whorl, *AG* works with the two B-class genes (*AP3* and *PI*) together to develop the stamens, while in the fourth whorl *AG* works alone to promote carpel development. In *Spinacia oleracea*, the *AG*

ortholog of spinach, *SpAG*, is only expressed in the primordia of the reproductive whorl (either stamen or carpel) when the first whorl differentiated (Sather et al, 2005). In *Arabidopsis*, when *AG* loses its functionality, the third whorl stamens are converted into petals (Meyerowitz et al. 1991). However, in the fourth whorl, the carpels are converted into sepals, rather than petals, inside which numerous extra floral whorls occur to form "new flowers" (Meyerowitz et al. 1991). Accordingly, *AG* seems more than just a *FHG*, and so has been termeda 'cadastral gene' that controls the expression boundaries of the A-class *AP1* and *AP2* (Irish and Sussex, 1990; Meyerowitz et al. 1991; Weigel and Meyerowitz, 1994). Thus, when *AG* loses its functionality, ectopic A-class expression occurs in the normal flowers, the third whorl turns to petals (A- and B-class genes act together) and the fourth whorl to sepals (A-class gene acts alone).

Second, the *AGAMOUS* gene seems to regulate floral meristem determinacy. In wild type *Arabidopsis*, a single floral meristem develops into one single flower, but as stated before, multiple whorls and flowers develop inside the fourth whorl when *AG* loses its function. Thus *AG* could terminate the floral meristem when the spatial pattern of floral whorls is determined, and as the formation of extra whorls needs extra cells, this behavior demonstrates that *AG* could negatively regulate cell proliferation. (Meyerowitz et al, 1991).

However, the two observed phenotypes in an *AG* loss of function mutant may not have to occur together. In *Arabidopsis,* the large (3.8 kb) second intron of *AG* controls whorl specific expression (Deyholos and Sieburth, 2000). Deyholos and Sieburth (2000) generated beta-glucuronidase (*GUS*) reporter

gene constructs from various fragments of the *AG* second intron, many of which showed differential GUS staining. Certain constructs were expressed predominantly in the third whorl while others were expressed predominantly in the fourth whorl. From this work it appears that *AG* contains whorl specific regulatory elements such as in the *AG* second intron. Therefore, the *AG* function loss appears only in a particular whorl since mutations may occur in either of the whorl specific regulatory sequences. But female *F. virginiana* flowers appear to have no conversion of carpels to sepals, nor do "new flowers" develop inside the fourth whorl, which means that the *AGAMOUS* gene seems not to be responsible for the female trait in *F. virginiana*.

Considering all of this, the B- and C-class floral homeotic genes may not be the best candidates for the observed phenotype in female strawberry flowers. In *Arabidopsis*, most of the Floral Homeotic Genes' natural alleles, or those derived through mutagenesis, are recessive. This would not disqualify them as candidates in a diploid organism, but *Fragaria virginiana* is an octoploid, containing eight sets of chromosomes. If we assume that all four homologous chromosome pairs contain copies of any given FHG, eight mutated copies of a gene are still highly unlikely to exist in a plant, making it highly unlikely that a recessive trait could be expressed.

On the other hand, we can also fairly assume an FHG gene could be present only in one subgenome but not in the others, thus a single mutant allele could exert a dominant negative effect in an octoploid. To address this question, we could use octoploid sequence data sets to determine gene copy number in

octoploids. However, this work will not be addressed in this project and could be done in the future.

Furthermore, it is well documented that femaleness is a dominant trait and females must be heterozygous for the sex-determining gene (Ahmadi and Bringhurst, 1991). Because the stamens are non-functional or absent, a female cannot be crossed either with itself or any other female; therefore pollen can only come from a male or hermaphrodite flower, preventing the occurrence of an FF genotype. The hermaphrodite flowers (H/H or H/M), when selfed, cannot produce females, but only generate hermaphrodites (when they are H/H) or hermaphrodites and males (when they are H/M) (Ahmadi and Bringhurst, 1991). Thus, females can only be heterozygous, so femaleness must be a simple dominant trait.

In sum, although some of the known *Arabidopsis* mutant phenotypes associated with the ABC Floral Homeotic Genes appear to be mimicked by female *Fragaria virginiana* flowers, many are not. Even when a similar phenotype was exhibited, it still could not be concluded that one of the B- or C-class Floral Homeotic Genes is responsible for sex determination in strawberry, since most mutant FHG alleles are recessive (while femaleness is dominant) and that *F. virginiana* is octoploid. Thus, it follows that another class of genes may take charge of the sexual determination in *Fragaria*. But, considering that the *AGAMOUS* gene has been isolated from strawberry (*Fragaria* × *ananassa*) and its expression has been well characterized (Aharoni et al. 1999; Rosin et al., 2003), it lends itself to be a candidate gene even though not the best.

The additional proposed candidate genes are as follows:

***SUPERMAN* Genes**

The *SUPERMAN* genes (*SUP*) are members of the *SUPERMAN*-like *RBE-SUP* sub-family of a larger family of proteins known as the C2H2 zinc-finger proteins (ZFP's) (Takeda, 2004; Hiratsu et al. 2004; Dathan et al., 2002; Englebrecht et al., 2004; Wolfe et al. 2000; Takatsuji and Matsumoto, 1996). *SUPERMAN* genes are referred to as "cadastral" genes, because they control the expression boundaries of floral homeotic genes in the various flower whorls, as opposed to the Floral Homeotic Genes themselves, like the ABC class of MADS-box genes, which determine the identity of floral organs and convert one floral organ or part into another type when malfunctioning (Bowman et al., 1992). Like the FHGs, the *SUPERMAN* genes are transcription factors, meaning that they regulate the expression of other genes.

Considerable pioneering research has been done with the model organism *Arabidopsis thaliana* to study *SUP* phenotypes derived from overexpression, ectopic expression, and loss-of-function (Kater et al., 2000; Yun et al., 2002; Sakai et al., 1995; Bereterbide et al., 2001). Furthermore, Schultz et al. (1991) found that *Arabidopsis SUP* loss-of-function mutants displayed supernumerary stamens and stamenoid carpels in the fourth whorl at the expense of female fertility, while the other three whorls remained phenotypically normal. Further investigations by Bowman et al. (1992) showed that the supernumerary stamen phenotype was not due to an extra amount of stamens formed in the third whorl, but because five more rings of stamen primordia had

developed in the fourth whorl. Also in *Arabidopsis SUP* loss of function mutants, the integuments grew equally on all sides of the ovule rather than growing asymmetrically to cover the ovule's abaxial side more than the adaxial side, and therefore severely reduced female fertility (Gaiser, 1995).

Overexpression and ectopic expression of the *SUP* gene in *Arabidopsis* resulted in a dwarf phenotype, which lacked proper cell proliferation, and had smaller flowers with few or no stamens (Yun et al, 2002). Kazama et al. (2009) have studied the putative *SUPERMAN* ortholog from *Silene latifolia*, designated *SlSUP*. By the smut fungus infection system (Alexander, 1990), a *35S::SlSUP* overexpression construct was introduced into *Silene latifolia* and the transformants produced fewer and smaller stamens, which is similar to the Yun et al (2002) results. Bereterbide et al. (2002) generated *35S::SUP* overexpression tobacco plants, and those plants were smaller than wild type tobaccos. Nakagawa et al. (2004) introduced *35S::PhSUP1* construct into wild type petunia, and found that transgenic plants exhibited a dwarf phenotype. However, the Kazama et al. (2009) plants were normally sized, and the authors estimated that this difference might be due to the use of a heterologous gene source. If the native *Arabidopsis* gene had been overexpressed, dwarfism would have been expected; however here, a non-native gene apparently had no effect on size. Additionally, methylation at the *SUP* locus has also been shown to have a significant impact on the number of stamens produced in the *Arabidopsis* flower (Jacobsen and Meyerowitz, 1997; Huang and Ma, 1997; Rohde et al., 1999): the more methylation an allele has, the more stamens will be produced,

which in turn lead to more cell expansion, division and differentiation. Accordingly, it is understood that *SUP* serves as a negative regulator of cell expansion, division and differentiation (Kater et al., 2000; Yun et al., 2002; Sakai et al., 1995; Bereterbide et al., 2001; Kazama et al. 2009; Nibau et al, 2010,). A corresponding impact on these processes would be seen when *SUP* malfunctions such as causing dwarfism when overexpressed.

The role of *SUP* genes in flower development is reasonably clear now. Many researchers have concluded that the function of the *SUP* gene products is to preclude the expression of B-class floral homeotic genes in the fourth whorl (Shultz, 1991; Bowman, 1992; Sakai, 1995). When there is a *SUP* loss of function, an expansion of B-class genes (*AP3* and *PI*) expression into the fourth whorl occurs and the flower develops extra stamens at the expense of fourth whorl carpels, whereas increased *SUP* expression reduces the expression of B-class genes. Yun et al. (2002) found that *Arabidopsis* plants overexpressing an *AP1*::*SUP* construct had severely reduced third whorl organs and enlarged fourth whorl carpels. It is believed that ectopic *SUP* expression inhibits the B-class gene expression to cause this phenotype since a reduction of *AP3* expression in the flowers was observed (Yun et al, 2002). Furthermore, compared to wild type *Arabidopsis*, *AP3* in *AP1*::*SUP* mutants expressed more slowly and in a relatively constricted area in the secondand third whorls (Yun et al, 2002).

Based on these studies, the function of *SUP* has been established: *SUP* expression helps to enforce the developmental boundary between the third and the fourth whorls by restricting B-class expression in the fourth whorl. However,

the mechanism of this regulation is still controversial, and has been addressed by two hypotheses. One model proposes that, either directly or indirectly, *SUP* initiates a signal at the third and fourth whorl boundary that precludes B-class homeotic gene expression in the fourth whorl (Bowman, 1992; Sakai, 1995; Smyth et al. 1990). But it is unclear whether *SUP* prevents the expression of *AP3*, *PI*, or both genes. As both B-class genes are needed for proper stamen development, the restriction of either *AP3* or *PI* could preclude stamen development in the fourth whorl. Another model hypothesizes that *SUP* controls the developmental boundary between whorls three and four by controlling spatial development between these two whorls, that is, cells in whorl 3 would not expand into whorl 4 and *vice versa* (Sakai et al., 2000).

Meyerowitz (1997) found that the *SUP* loss of function mutant phenotype was caused by continued cell proliferation in the third whorl cells and lack of cell division and expansion in fourth whorl cells, supporting the second hypothesis. It is well established that *SUP* is a negative regulator of cell division and expansion. And then, Sakai et al. (2000) proposed that the increase in stamens in *SUP* loss of function mutants is not due to the fourth whorl carpels being converted to stamens but due to the oversized third whorl which developed redundant stamens and expanded at the expense of the fourth whorl. Bereterbide et al. (2002) performed convincing research that demonstrated the action of *SUP* in maintaining appropriate levels of cell division and expansion. The fertility of a cytoplasmic male sterile (CMS) tobacco line was caused by uncontrolled growth of the third and fourth whorls such that the carpels and stamens became fused

and formed male sterile stamen/carpel mosaic organs. Bereterbide et al. (2002) transformed an overexpression construct of *Arabidopsis SUP* into a CMS (cytoplasmically male sterile) tobacco line. With the amounts of *SUP* gene product restored, the cell proliferation was diminished, and the stamens became phenotypically normal and produced functional pollen. Thus, the *SUP* gene does not preclude the expression of B-class genes in the fourth whorl, but restricts the third whorl spatial development to prevent it from expanding into and consuming the fourth whorl. Note that third whorl expansion can only be accomplished at the expense of the fourth whorl (Sakai et al., 2000).

The research on overexpression of *SUP* suggested that the second model, where the *SUP* gene restricts spatial development into the third whorl, would be the most plausible. The overexpression of *SUP* should suppress cell spatial development, thus it is easy to see *SUP* controlling the developmental boundary between whorls three and four through spatial development rather than by controlling gene expression. In fact, the overexpression of *SUP* would cause a dwarf phenotype (Bereterbide et al., 2002; Hiratsu et al. 2002; Nakagawa et al., 2004). It is also of interest to note that in *Arabidopsis SUP* loss of function mutants, *AP3* expression is not found in the center of the fourth whorl (Bowman et al., 1992). This result seems to support the second model that *SUP* controls cell expansion from the third whorl into the fourth whorl, as it indicates that the very center of the flower retains its wild type fourth whorl identity. If the first model, where the *SUP* gene prevents the expression of B-class genes in the

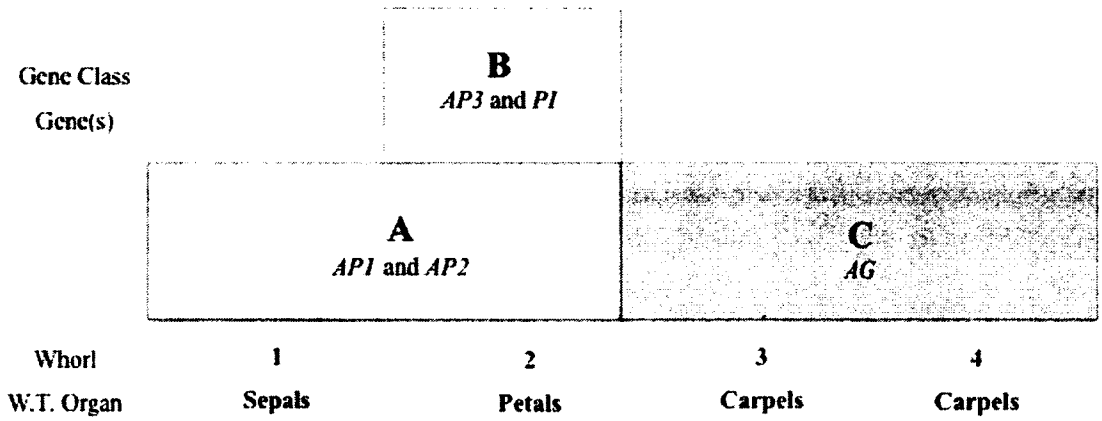
fourth whorl, were true, it would be expected that *AP3* would express in all tissue interior to the third whorl.

RABBIT EARS

RABBIT EARS (*RBE*) is another member of the *SUPERMAN*-like *RBE-SUP* sub-family. It is a transcription factor that acts as a cadastral gene and controls the boundary between whorls two and three in *Arabidopsis* flowers (Takeda et al., 2003). But compared to *SUPERMAN* genes, relatively little is known about *RBE*. Krizek et al. (2006) found that *RBE* could preclude the expression of *AGAMOUS* (*AG*) in the second whorl. In *RBE* loss of function flowers, *AG* is expressed in the second whorl, and suppresses the second whorl from growing (Bowman et al., 1991). This mechanism is further supported by the fact that *RBE/AG* double loss of function mutants produce normal petals in whorl two. Therefore, the *RABBIT EARS* gene, unlike the *SUPERMAN* gene, seems to be a positive regulator of growth in the flower. But Takeda et al. (2003) generated lines of 35S::*RBE Arabidopsis* and found that transgenic plants were phenotypically normal. *SUP* and *RBE* both act to prevent Floral Homeotic Gene expressin from exceeding the particular whorls, thus ectopic or overexpression of these genes would have similar effects. When *SUP* was ectopically expressed (Yun et al., 2002), expression of B-class genes was restricted in the third whorl (Figure 3a). Following this line of thought, ectopic or overexpression of *RBE* would prevent *AG*, the C-class gene, from expressing in the third whorl and stop proper stamen development (Figure 3b), thus producing a female flower

As stated above, the genetic control of flower development is not as simple as the ABC model proposes. Flower development requires precise controls of the ABC genes. One of these controls is to confine particular FHGs to express in a certain whorl. *SUPERMAN* genes and *RABBIT EARS* serve as cadastral genes, and create FHG expression boundaries between whorls, thus they are essential to flower development.

a. *SUP* O.E.



b. *RBE* O.E.

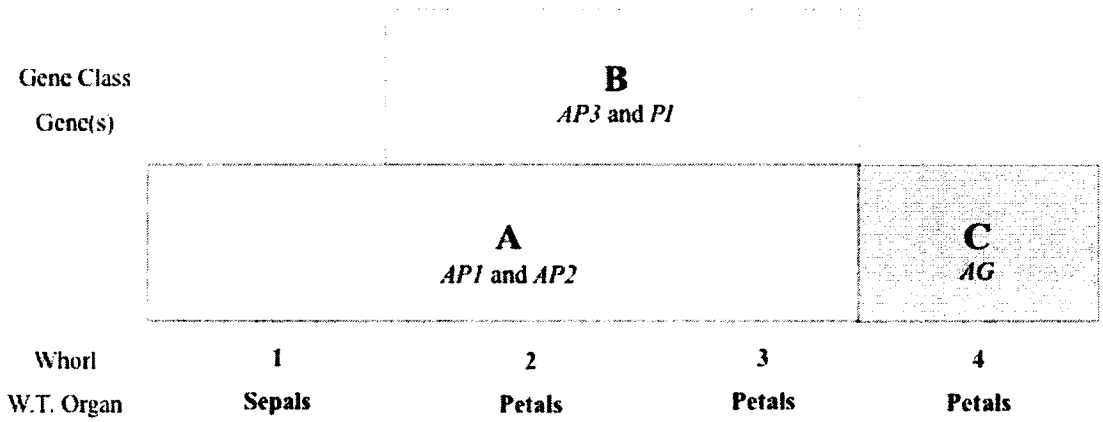


Figure 3. Phenotypic consequences of *SUP* and *RBE* overexpression (O.E.). **a.** With extra *SUPERMAN* genes product, *AP3* and *PI* are removed from whorl 3. This results in fewer stamens, undeveloped stamens or stamen/carpel mosaic organs in whorl 3. **b.** With extra *RABBIT EARS* gene product, *AG* is removed from whorl 3. This allows *AP1* and *AP2* to occupy whorl 3, converting the organs to petals, which cause fewer stamens, undeveloped stamens or stamen/carpel mosaic organs in whorl 3.

Ethylene and Sex Determination: The *ACS-7* Gene

Not only do transcription factors control flower development, but also some small gaseous molecules exert control over flower development. It is known that ethylene is a potent phytohormone. In the 1950's, one role of ethylene as a plant hormone was found to be the promotion of female flower development in cucumber (Boualem et al., 2008). Exogenous ethylene could suppress the development of stamens and in turn promote the development of female organs. Thus, the gene involved in synthesis of ethylene plays a direct role in the suppression of stamen development.

The genus *Cucumis* (melon and cucumber) represents an astonishing variety of sex morphs, with four reproductive strategies shown in this genus (Boualem et al., 2008). It may be monoecious (separate male and female flowers on the same plant), andromonecious (male and hermaphrodites on the same plant), gynoecious (only female flowers), and hermaphrodite (perfect flowers). All four sex types are known to exist in *C. melo* (melon), with andromonecy under the control of the "A" locus, which has been cloned and characterized. The results show that this locus contains an *ACS* gene that displays the highest degree of homology to the *Arabidopsis ACS-7* gene. The latter gene codes for 1-aminocyclopropane-1-carboxylic acid synthase, a rate-limiting enzyme in ethylene biosynthesis (Yamagami et al, 2003). Thus, the *Cucumis melo* gene was designated *CmACS-7*.

The *CmACS-7* activity assay provided direct evidence that differing ethylene production may affect sex (Boualem et al., 2008). The *CmACS-7* genes

from 496 *C. melo* accessions of differing sex type were sequenced. It was found that all monoecious and gynoecious plants (n = 149) contained the active *CmACS-7* while the andromonoecious and hermaphroditic plants (n = 347) did not. The model suggested by these data that plants able to make female flowers (monoecious and gynoecious) have the active *CmACS-7* while all plants that cannot (andromonoecious or hermaphrodite) lack this active gene. From the work presented by Boualem et al. (2008), it is clear that in *Cucumis melo* ethylene is either required for or the direct cause of stamen suppression.

Therefore, *ACS-7* is an intriguing candidate gene for sex determination in *Fragaria virginiana* since it is highly correlated with the suppression of stamen development in melon.

1.6 Initial Work

UNH graduate student Ben Orcheski (2010), my predecessor on this project, isolated the candidate genes described above from strawberry, and built both RNAi and overexpression constructs through Gateway cloning. Then, he transformed those constructs into *Arabidopsis thaliana* ecotype Colombia-0 plants by the floral dip method (Clough and Bent, 1998), and confirmed that transformants contained inserts. However, no phenotypes were reported. Thus, to transform these constructs into strawberry was the next logical step.

1.7 Choice of Diploid *Fragaria vesca* as the Subject of Investigation

The cultivated octoploid strawberry is interesting, but its genomic complexity complicates the interpretation of genetic transformation studies. Use of an appropriate diploid relative was expected to increase the ease of genetic transformation and subsequent analysis.

Fragaria vesca

F. vesca (Figure 4) was used throughout this study to transform constructs of candidate genes. *F. vesca* ($2n = 2x = 14$, also known as the woodland strawberry, has the most geographical diversity among all strawberry species, growing naturally in Asia, Europe, North and South America. Due to its extensive range, several subspecies of *F. vesca* exist (Bors and Sullivan, 2005).

F. vesca has become the model species for functional genetics and genomics in the strawberry community for many reasons. First, *F. vesca* is a diploid, making it genetically much less complex to work with as compared to the octoploid species. Second, it is self-fertile which makes it more likely to develop inbred homozygous lines for transformation. Additionally, the 'Alpine' forms of *F. vesca* are easily propagated by tissue culture (Oosumi, 2005), making it an ideal plant for genetic transformation.

In this study, the *F. vesca* ssp. *vesca* 'Hawaii-4' was used for two reasons. First, the 'Hawaii-4' genome has been sequenced (Shulaev et al. 2011), and therefore we could isolate genes from it. Second, an efficient *Agrobacterium* mediated transformation system has been established using *F. vesca* variety Hawaii (Oosumi, 2005).

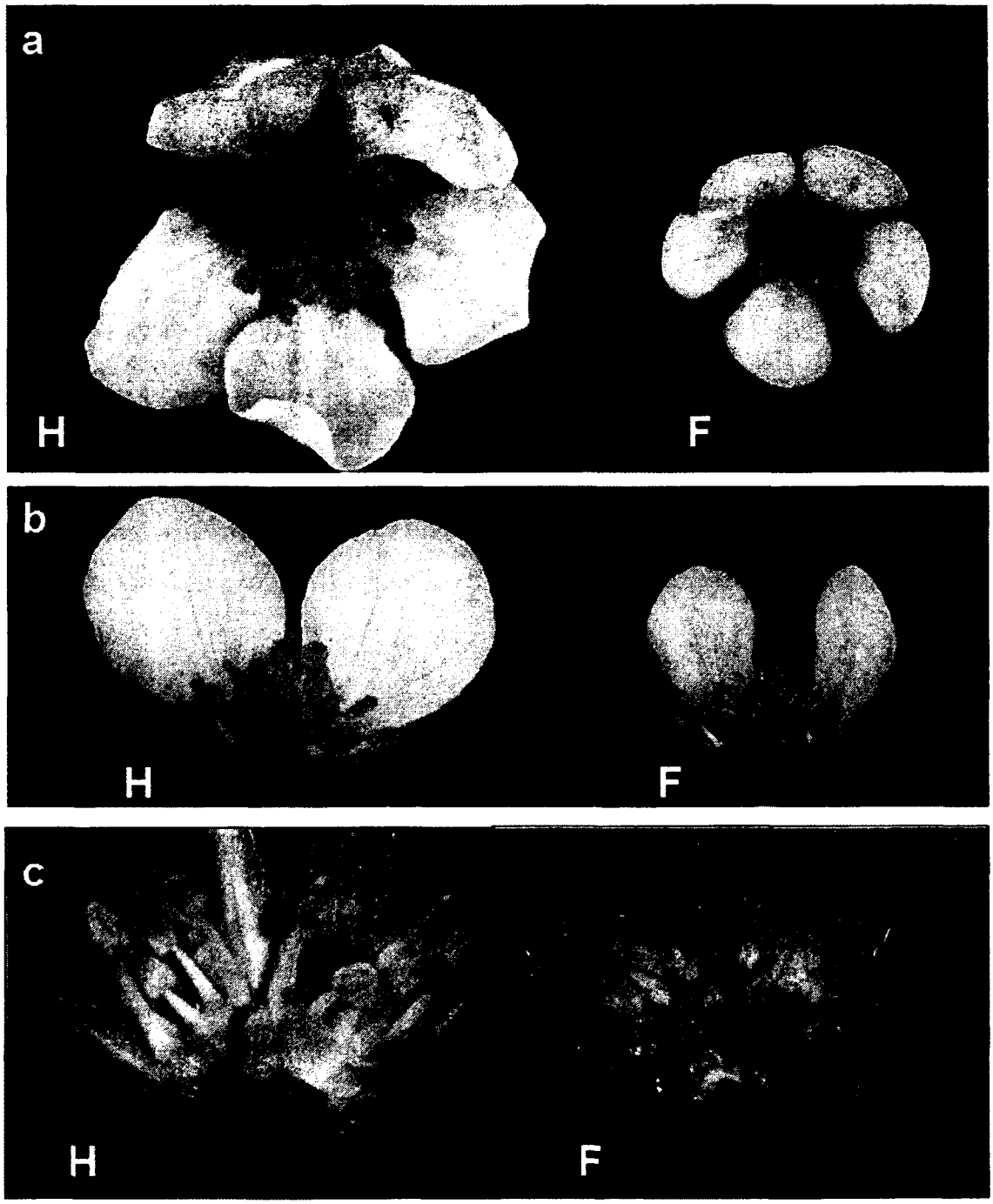


Figure 4. Morphology of hermaphrodite and female *F. virginiana* flowers. **a.** Top view of fully opened hermaphrodite (H) and female (F) flowers. **b.** Lateral view of the flowers as dissected through the center. **c.** Mature hermaphrodite and female flowers with receptacle removed. Note the difference between H and F in the size of the flowers (**a** and **b**), and of the stamens (**c**). From Orcheski (2010).

1.8 Research Goal

Although the previous works have shown informative results in *Arabidopsis*, the phenotypes generated by overexpressing or knocking out each of the seven chosen candidate genes will give a better understanding of whether and how they are involved in flower development in strawberry. To discover genes whose phenotypes mimic those of female *F. virginiana* flowers, namely vestigial stamens, would make a valuable contribution to the field of strawberry genetics. Hence, this project primarily aimed at determining the phenotypes resulting from introducing each of seven candidate genes into diploid strawberry. These included five strawberry *SUP* homologues, and strawberry *AGAMOUS* and *ACS-7* homologues.

CHAPTER 2

MATERIALS & METHODS

Some materials used in this project were the products of previous work by Benjamin Orcheski (2010), as indicated below.

2.1 Elements of the Destination Vector

The two destination vectors used in this study, pH7WG2D and pK7GWIWG2D(II), were constructed by Benjamin Orcheski (2010) in his Master's Thesis research. They are based on the pPZP200 plasmid backbone (Hajdukiewicz et al., 1994), which has two origins of replication (one for *E. coli* and one for *Agrobacterium*), the left and right T-DNA border sequences needed for gene transfer, and a selectable marker – the spectinomycin resistance gene. Beside these common elements, each type of destination vector contains its own specific elements.

The pK7GWIWG2D(II) vector (5a) is an RNAi vector, from which a double-stranded RNA of the candidate gene (or gene segment) is produced, for the purpose of silencing the corresponding native gene's expression. To do this, two copies of the candidate gene (or gene segment) must be inserted into the vector in opposite orientations and separated by a short spacer sequence. The *35S* promoter drives the transcription of the two candidate gene copies in a single transcript which forms a stem loop of dsRNA. When this dsRNA is then processed by the RNAi pathway, the anticipated result is to silence the native

gene. The pK7GWIWG2D(II) vector contains a kanamycin resistance gene to allow selection of cells/plants that have incorporated the introduced construct.

In contrast, the pH7WG2D vector (Figure 5b) contains a *35S* constitutive promoter that can constitutively express the inserted candidate gene sequence, which is present in a single, complete copy in forward orientation. The intent of this construct is to cause an overexpression phenotype when introduced into plants. In addition, this vector uses the hygromycin resistance gene to select cells/plants that have been transformed with the overexpression construct.

2.2 Bioinformatic Confirmation of Insert Sequence Identity

The DNA sequence information for the seven candidate genes was obtained from Benjamin Orcheski (2010), and then each of these sequences was positioned at the appropriate position within the multiple cloning site(s) in each of the two destination vectors, RNAi and overexpression. By doing this, 14 different destination vectors sequences were reconstructed *in silico*. The sequences of PCR primers (Table 1) previously designed and provided by Benjamin Orcheski were aligned to the reconstructed vector sequences using PrimerSelect software (DNAStar) to confirm the direction and position of the primers in the constructs, and also to confirm that the primers did not have alternate (non-target) binding sites within the recombinant vectors and that they should make single, unique products when the isolated, recombinant vectors were used as PCR templates.

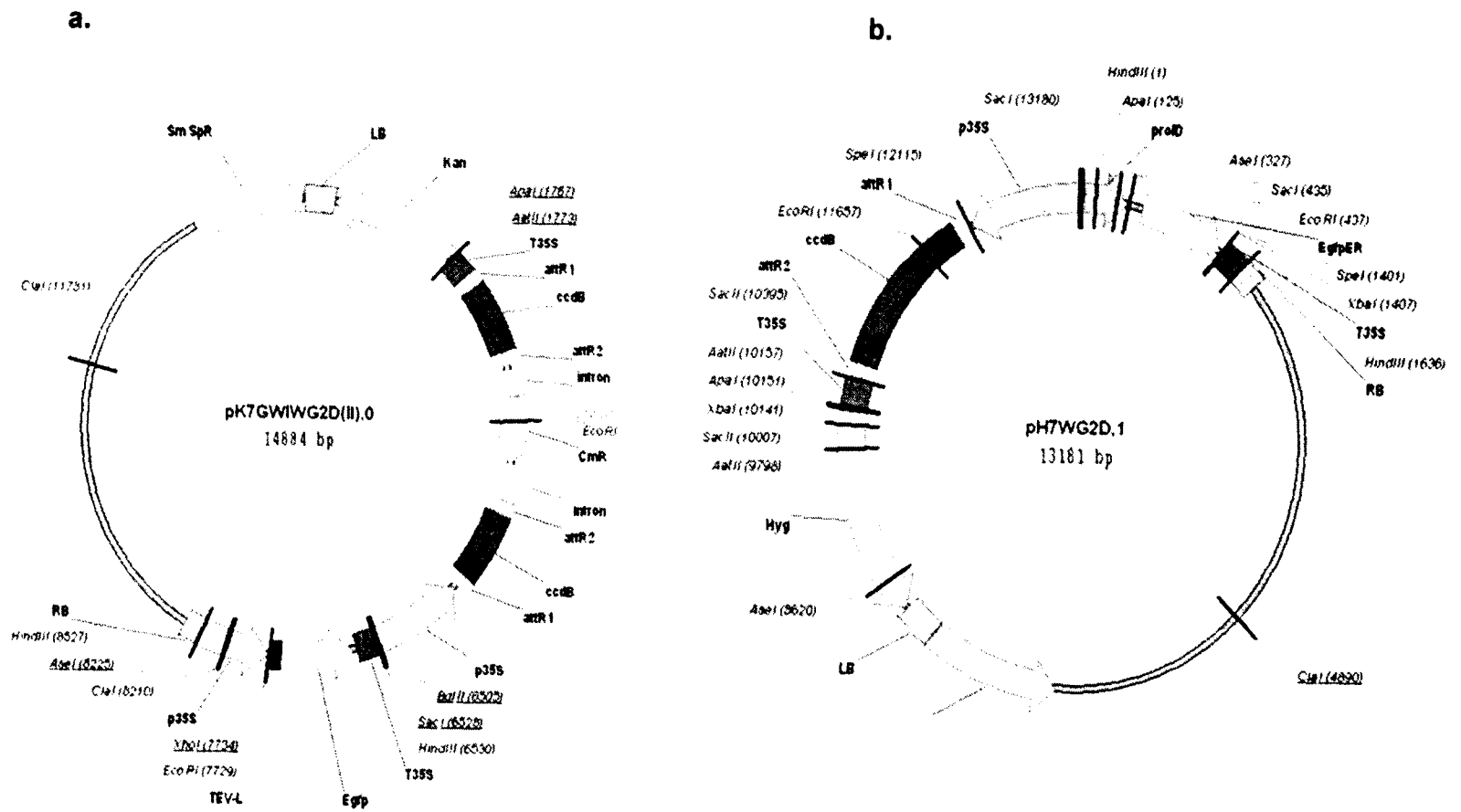


Figure 5. Plasmid Maps of the Vectors Used for Candidate Gene Transformation. Plasmids were obtained from VIB-Research (University of Ghent, Belgium). **a.** The pK7GWIWG2D(II) was used for RNAi studies. **b.** The pHWG2D was used for overexpression studies. Diagram courtesy of the University of Ghent.

2.3 Confirmation of *Agrobacterium* Clones

Each strain of transformed *Agrobacterium* made by Benjamin Orcheski, both overexpression and RNAi expression clones, was streaked on an LB agar plate containing 50µg/mL spectinomycin. Plates were incubated at 28°C for 24 hours. Individual *Agrobacterium* colonies were then isolated and screened for inserts by sub-culturing on spectinomycin plates followed by colony PCR. For the latter purpose, liquid cultures were prepared by transferring a picked samples from a single colony into 6 ml LB liquid medium (10g/L tryptone, 10g/L NaCl, 5g/L hydrolyzed yeast) containing 50µg/mL spectinomycin, and incubating overnight at 30°C while shaken at 250 RPM. PCR reactions were prepared as follows: 25µl reactions were prepared using 2.5µl of 10X AccuPrime Buffer II (Invitrogen, Grand Island, NY), 0.4µM of both F Trans and R Trans primers for each gene (see Table 1), 0.5U High Fidelity Taq DNA polymerase (Invitrogen) and 60ng of template DNA, run using a profile with an initial denaturation at 94°C for 1 minute followed by 30 cycles at 94°C for 30 seconds, 52°C for 30 seconds and 68°C for 30 seconds per 500 bp (expected product size), and ending with a final extension at 68°C for 10 minutes. Once the presence of an insert of the expected size was confirmed, a single positive colony was then sub-cultured by streaking it on an LB agar plate (10g/L tryptone, 10g/L NaCl, 5g/L hydrolyzed yeast, 15g/L agar) containing 50µg/mL spectinomycin. Plates of confirmed clones were incubated at 28°C for 24 hours, and then individual *Agrobacterium* colonies were picked for *Agrobacterium* transformation.

Table 1. Primer Sequences Used in This Study. Products amplified by the primers span from the start to stop codon of each candidate gene and include attB adaptor sequence at the primers' 5′ end (* indicates primer obtained from Benjamin Orcheski; attB1and attB2 adaptor sequences are underlined with straight (___) or wavy (___) lines, respectively).

Transformation Primers	Primer Sequences
Universal attB1 Universal attB2	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′ 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′
*FRASUP1 F Trans *FRASUP1 R Trans	5′-AAAAAGCAGGCTATGATGGATTGTGTTAAGTT-3′ 5′-AGAAAGCTGGGTCTAGAGATGCCCGAGTCGAA-3′
*FRASUP2 F Trans *FRASUP2 R Trans	5′-AAAAAGCAGGCTATGAACACTGCTCGCTTCTCG-3′ 5′-AGAAAGCTGGGTTTAGCGTAATGAATCGTCTA-3′
*FRASUP3 F Trans *FRASUP3 R Trans	5′-AAAAAGCAGGCTATGCAGAGGAACAGTGGCTC-3′ 5′-AGAAAGCTGGGTTCAGTAGCCTAATCGAAGCT-3′
*FRASUP4 F Trans *FRASUP4 R Trans	5′-AAAAAGCAGGCTATGCTTGAAGGAGACTGCTA-3′ 5′-AGAAAGCTGGGTTTAGCTTCTTCTTCTTCTT-3′
*FRASUP5 F Trans *FRASUP5 R Trans	5′-AAAAAGCAGGCTATGGAGCAAGCACGGTACTG-3′ 5′-AGAAAGCTGGGTTTAATCTACTTTGATTACCT-3′
ACS7 F Trans ACS7 R Trans	5′-AAAAAGCAGGCTATGGCTATAGAGATTGAGCA-3′ 5′-AGAAAGCTGGGTCTACTCCCTTGTCTTCTTC-3′
*AG F Trans *AG R Trans	5′-AAAAAGCAGGCTATGGCCTATGAAAACAAACC-3′ 5′-AGAAAGCTGGGTTTACACTAACTGAAGGGAAA-3′
p35SF	5′-ATGAAGACGATGTACCCGACTAATAAT-3′
AG SR	5′-GGGCATCAGCGTCCAGGTCAGT-3′

2.4 Preparation of Explants Material for Transformation

Newly unfolded trifoliate leaves were excised from 'Hawaii-4' plants that were growing in the greenhouse. After excision, leaves from those plants were immediately surface-disinfected with 70% alcohol pads (Dynarex, Orangeburg, NY), then the leaves were separated into leaflets and thoroughly washed under running tap water for 1 hour. The rest of this protocol was performed in the hood. Leaflets were transferred into 70% ethanol for 1 min, and finally agitated gently in 1% sodium hypochlorite for 8 min. After this surface sterilization procedure, the leaflets were rinsed five times with sterile water. The surface-sterilized leaflets were placed abaxial side up on four layers of sterile filter paper moistened with sterile water in the lid of a Petri dish. Using a sterile scalpel, the petiole and midvein of leaflets were removed, and leaf blades were cut diagonally across the secondary veins into 10-15 × 10-15mm rectangular pieces. After sectioning, the explants were blotted dry on sterile filter paper and transferred, abaxial side up, to a Petri dish containing co-cultivation medium (CCM) (4.44g/L MS salts and B5 vitamins, 2% sucrose, 3mg/L N6-benzyladenine (BA), 0.2mg\L indole-3-butyric acid (IBA), 0.7% agar, pH 5.5). Plates were sealed with Parafilm and incubated at room temperature in the dark for 3 days prior to use in co-cultivation treatments (described below).

2.5 Agrobacterium Culture

For each transformation experiment, a single confirmed positive *Agrobacterium* colony for each construct was incubated in 2ml LB liquid medium

(10g/L tryptone, 10g/L NaCl, 5g/L hydrolyzed yeast) containing 50µg/mL spectinomycin in a 15ml conical tube for 22 hours at 30°C on a shaker (250 RPM).

2.6 Plant Transformation

For each treatment, 1 ml of the *Agrobacterium* culture was centrifuged at 15,700 x g for 3 min and the supernatant was discarded. Then the pellet was re-suspended in 2ml MS + B5 medium [MS basal salts, B5 vitamins, 2% sucrose, pH 5.5] and raised to the final volume of 20ml MS+B5 medium in a deep Petri dish. The explants were placed abaxial side up on four layers of sterile filter paper moistened with MS + B5 medium in the lid of a Petri dish, and cut diagonally across the secondary veins into 1.0–1.5mm wide pieces. The explants were placed into the *Agrobacterium* suspension immediately after cutting. The dishes were incubated for 20 min at room temperature and agitated gently by hand every 5 min in the hood. After incubation, the explants were blotted dry on sterile filter paper and transferred, abaxial side up, to a Petri dish containing 20ml CCM.

The plates were sealed with Parafilm and incubated at room temperature in the dark for 3 days. After incubation, to remove *Agrobacterium*, the explants were rinsed twice in washing-off medium (WOM) (20ml MS + B5 medium with 500 mg/L carbenicillin) in 50ml conical tubes, and vigorously vortexed. The WOM was discarded and replaced with 30ml fresh WOM solution. The tubes were

incubated at room temperature for 30 min and shaken gently every 5 min. The WOM was again discarded and the explants were rinsed with 10ml fresh WOM briefly, and then blotted dry on sterile filter paper. After washing, the explants were transferred, abaxial side up, to non-selective shoot induction medium (SIM) (4.44g/L MS salts and B5 vitamins, 2% sucrose, pH 5.8, 3mg /L BA, 0.2mg/L IBA, 500mg/L carbenicillin, 0.7% agar). The carbenicillin is intended to kill the residual *Agrobacterium* cells. The plates were incubated in a growth room (25℃, humidity 70%) under 16 h photoperiod at light intensity of 20–30 μM m^{-2} s^{-1}.

After 1 week, the explants were transferred to selective SIM containing 250mg/L carbenicillin and 4mg/L hygromycin B (overexpression) or 50mg/L kanamycin (RNAi). Before transfer to SIM medium, the primary explants were cut into three or four secondary explants by dividing them between the veins so that each secondary explant was bisected by a secondary vein. After another week of culture, the explants were transferred to fresh selective SIM and subcultured every 2 weeks.

Transgenic calli and shoots were screened for GFP expression 7 weeks after infection for overexpression constructs, and 8 weeks for RNAi constructs. The GFP (+/-) phenotype of each explant with calli or shoots was recorded and then all explants were transferred to non-selective SIM containing 100mg/L carbenicillin. The explants were incubated under the same conditions,subcultured every 4 weeks, and periodically examined for presence of developing shoots. Well-developed shoots were placed into deep Petri dishes

containing 40ml root induction medium (RIM) (4.44g/L MS salts and B5 vitamins, 2% sucrose, pH 5.8, 250mg/L carbenicillin, 0.7% agar). Rooted plants were transferred to Magenta vessels containing 50ml RIM. After varying length of incubation, depending on the rate of root growth, the plants were transplanted to 6-inch growth pots in greenhouse, and grown on until ready for genotyping.

2.7 Fluorescence Microscopy

Visualization of GFP fluorescence in plant tissue was done using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Inc., New York, NY) with a 100 W mercury burner (Olympus BH2-RFL-T3) and filter sets for GFP longpass, narrow-band GFP, and FITC/TRITC (filter sets no. Fset01, □Fset09, and Fset15, respectively).

2.8 Genomic DNA Extraction from Transformants

From each transformant, 0.1g fresh unexpanded strawberry leaves were collected and gently ground in liquid nitrogen until the tissue turned to fine powder. Then, 1ml grinding of buffer (2% CTAB solution with 0.4% β-Mercaptoethanol) was added and continued grinding until the mixture became slurry, and then transferred the slurry to 1.5ml sterile microfuge tubes. Tubes were incubated at 60°C for 30 min and cooled at room temperature for 10 min, then 800µl 24:1 chloroform:octanol was added to each tube. Closed tubes were vortexed until the liquid was uniformly green, then centrifuged at 14,000xg for 5

min. The supernatant was transferred to a new sterile 1.5ml microfuge tube, 1ml of 95% ethanol was added and tubes were held in -20□ overnight. The following day, tubes were gently inverted several times and centrifuged at 14,000xg for 5 min and then the supernatant was discarded. Next, 1 ml cold 70% ethanol was added into the tubes and held in -20□ overnight, then centrifuged at 14,000xg for 5 min and discarded the supernatant. Residual ethanol was removed with pipette and air-dried in the laminar flow hood, and then added 25µl TE buffer. DNA was resuspended overnight and treated with 10µg/ml RNase A.

2.9 Confirmation of Transgene Presence in Regenerated Strawberry Plants

PCR Primer Design

For checking the inserts *in vivo*, new primers were designed to be specific to the chimeric vector:insert sequences. Using PrimerSelect software (DNAStar) and referring to construct maps (Figure 4), a universal primer, p35SF, was designed as a forward primer that annealed to the p35S site, which is a site specific to the vectors. For the reverse primers, differing strategies were chosen depending on the various lengths of each insert sequence. For constructs containing any of the six genes, *FRASUP1* to *FRASUP5* and *ACS-7*, we used the reverse (R) Trans primers (shown in Table 1), which aligned to the junction of the attR2 site and insert-specific sequence. For *AGAMOUS*, we designed a gene specific primer (AG SR) that annealed within the gene and, in partnership with

primer p35SF, produced a PCR product around 1 Kb in length. Primer positions are shown in Figure 6.

PCR Assays of Putatively Transformed Plants

PCR was performed to confirm that the transformant plants that survived the selective process harbored the particular candidate genes with which they had been transformed. Using the High Fidelity Taq protocol discussed above, 25µl PCR reactions were made for each plant to be assayed. PCR reaction mixes were prepared using the primers and templates shown in Table 2. The PCR profile for the amplification was an initial denaturation at 94°C for 1 minute followed by 30 cycles at 94°C for 30 seconds, 52°C for 30 seconds and 68°C for 30 seconds per 500 bp, and ending with a final extension at 68°C for 10 minutes. The products were visualized as describe above.

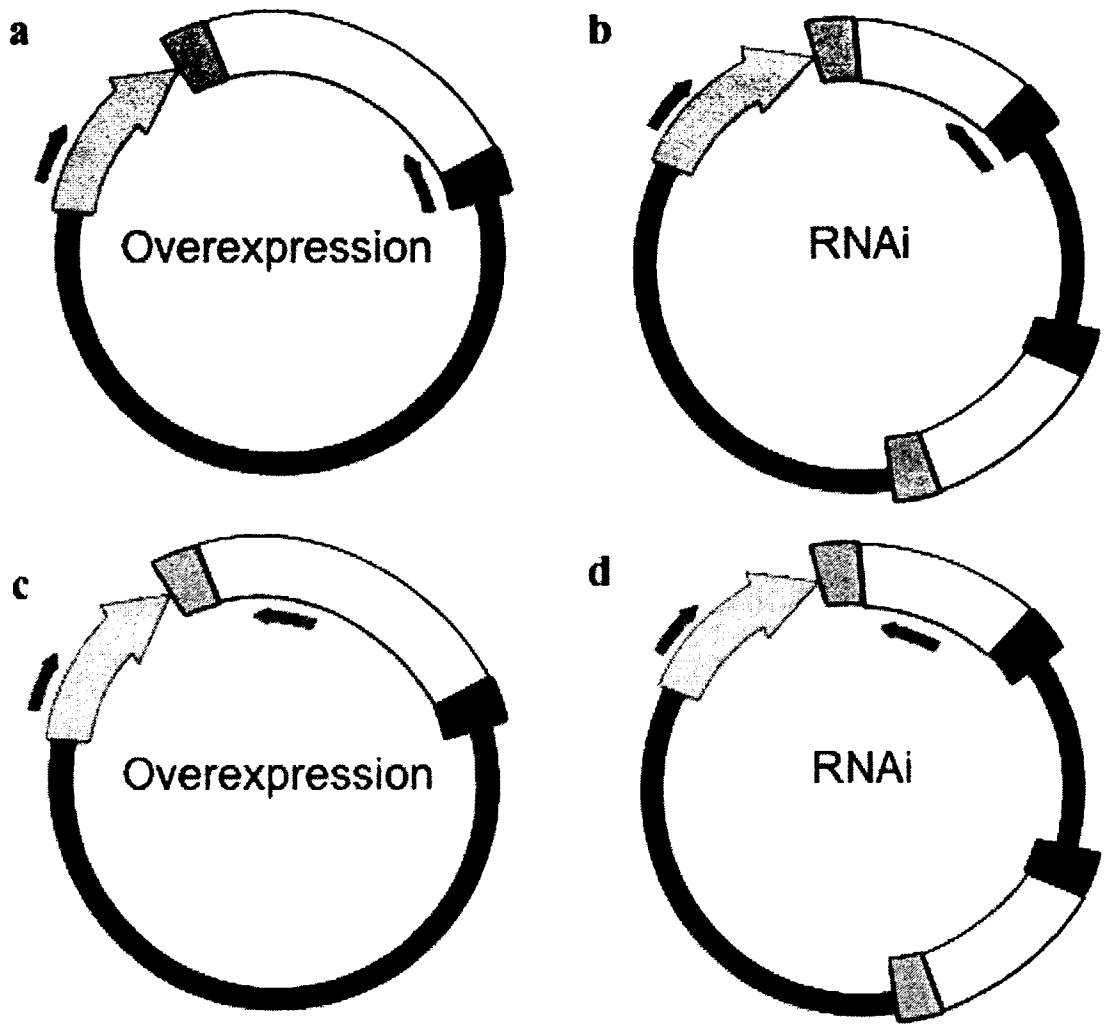


Figure 6. Positions of Primers Used for Confirmation of Candidate Sequence Transformation. The primers shown (orange arrows) were used for confirming the inserts in regenerated plants. Primer positions for *FRASUP1 to FRASUP5* and *ACS-7* are indicated in **a** and **b** above. Primer positions for *AGAMOUS* are indicated in **c** and **d** (attR2 site shown in green).

Table 2. Primers and Templates for Insert Assays

	Forward Primer	Reverse Primer	Template
Positive Control	p35SF	*	Plasmid
Negative Control	p35SF	*	# Genomic DNA
FRASUP 1	p35SF	FRASUP1 R Trans	§ Genomic DNA
FRASUP 2	p35SF	FRASUP2 R Trans	§ Genomic DNA
FRASUP 3	p35SF	FRASUP3 R Trans	§ Genomic DNA
FRASUP 4	p35SF	FRASUP4 R Trans	§ Genomic DNA
FRASUP 5	p35SF	FRASUP5 R Trans	§ Genomic DNA
AGAMOUS	p35SF	AG SR	§ Genomic DNA
AGS 7	p35SF	AGS 7 R Trans	§ Genomic DNA

* The reverse primer for positive and negative control is chosen appropriate to the gene that is being assayed.
** The plasmid used appropriate to the gene that was being assayed.
# DNA (8E8) was provided by Qian Zhang
§ Templates were obtained from the appropriate RNAi or overexpression transformed plants

2.10 Observation of Phenotypes

Following co-cultivation, all the explants were observed on a daily basis. For any explant that initiated callus, the first date of callus observation was noted. In cases where no callus was observed, regular observations were continued every one or two days until the whole explants dried and blackened, at which time they would be recorded as "dead". All newly formed calli were observed under a dissecting microscope (Olympus SZH-ILLD) daily to assess their growth and health, and any evidence of shoot formation was tallied. Any callus that failed to form shoots but turned to brown, black, or pale was documented with photographs and was still regularly sub-cultured and observed until 9 months later to confirm "it really was sincerely dead". Beginning with the 7^{th} week after cocultivation, calli were screened for GFP expression using confocal microscope, and both GFP^+ and GFP^- results were recorded. After that, all calli, shooted or not, were checked for GFP expression on weekly basis; however only shooted calli were transferred to RIM. All calli that did not form shoots were regularly checked, and subcultured and observed until categorized as dead. When regenerated shoots grew into plantlets, the GFP expression check was run every 10 days. All unrooted calli were tallied, subcultured, and observed until categorized as dead.

All rooted plantlets were transferred to 6-inch pots in the greenhouse and their growth was observed on a daily basis until they flowered. As soon as flowering started, each flower was observed by naked eyes to check for presence and approximate numbers of stamens and for overall floral morphology.

Once an abnormal phenotype was discovered, the flower(s) would be photographed and dissected under the dissecting microscope (Olympus SZH-ILLD).

CHAPTER 3

RESULTS

3.1 Confirmation of Expected Insert Sizes in Recombinant Plasmids

All 14 recombinant constructs were checked by PCR for presence of the appropriate insert using gene specific primers (Table 1). The results (Table 3) confirmed that the appropriate inserts were present in each of those 14 recombinant constructs.

3.2 Development of Transformed Explants

Although a response of some type by explant tissues was observed with all recombinant constructs following *Agrobacterium* co-cultivation, differences were seen among construct types with respect to the initiation and continued viability of the callus, and the presence/absence or timing of plant regeneration. Typically, as with RNAi (R) constructs *FRASUP1*-R, *FRASUP2*-R, *FRASUP4*-R, *FRASUP5*-R, *AGAMOUS*-R, and *ASC-7*-R, and overexpression (OE) constructs *FRASUP2*-OE, and *ASC-7*-OE, the initiation of swollen "nodes" on explants was observed 3~7 days after co-cultivation, then nodes gave rise to callus. The typical callus initially formed a yellow-white cell cluster, and then the green meristematic nodules formed on the calli and thereafter gave rise to shoots by 7 or 8 weeks after co-cultivation; and at shoot initiation, callus growth would cease.

Table 3. PCR Confirmation of Recombinant Plasmid Insert Sizes.

Construct	Expected Product Size	Observed Product Size	Negative Control Reaction
FRASUP1-R	660	680	No Product
***FRASUP1*-OE**	660	680	No Product
FRASUP2-R	579	600	No Product
***FRASUP2*-OE**	579	600	No Product
FRASUP3-R	672	700	No Product
***FRASUP3*-OE**	672	700	No Product
FRASUP4-R	780	830	No Product
***FRASUP4*-OE**	780	830	No Product
FRASUP5-R	954	1010	No Product
***FRASUP5*-OE**	954	1010	No Product
ACS-7-R	2588	2700	No Product
***ACS-7*-OR**	2588	2700	No Product
AGAMOUS-R	3225	3280	No Product
***AGAMOUS*-OE**	3225	3280	No Product

With these typical cases that resulted in shoot regeneration, callus initiation itself ceased after ~8 weeks culture.

In contrast, with overexpression constructs *FRASUP3-OE*, *FRASUP4*-OE, and *FRASUP5*-OE, the callus-producing nodes began to quickly degenerate one or two days after they were first noticeable (3 or 4 days after co-cultivation). Or in a different departure from the typical pattern, overexpression constructs *FRASUP1*-OE and *AGAMOUS*-OE and the RNAi construct *FRASUP3*-R produced calli that enlarged but grew only as callus until about the 16th week. Thereafter, these calli remained a constant size, and then started to partially turn off-white. By the 19th week, the white parts of these calli began to secrete gel-like liquid. The liquid secretion appeared to resemble a bacterial infection but did not behave like one, because bacterial infection would have extended outward from the calli onto the medium and finally form an opaque colony of pink or gray color. But instead, the gel-like liquid dried up during the next few weeks, while the calli turned light brown, then finally turned black. These degenerated calli were considered being dead after 24 to 25 weeks after co-cultivation. (Figure 7)

With constructs *FRASUP1*-R, *FRASUP2*-R and *FRASUP2*-OE, *FRASUP4*-R, *FRASUP5*-R, *AGAMOUS*-R, and *ASC-7*-R and *ASC-7*-OE, about one-third of the initiated calli eventually appeared dead, while the remaining two-thirds continued to grow and form shoots. These "shooted" calli were transferred to root induction media, where about 90% of these produced rooted plantlets, while the remaining 10% did not form roots and withered (Data in Table 4).

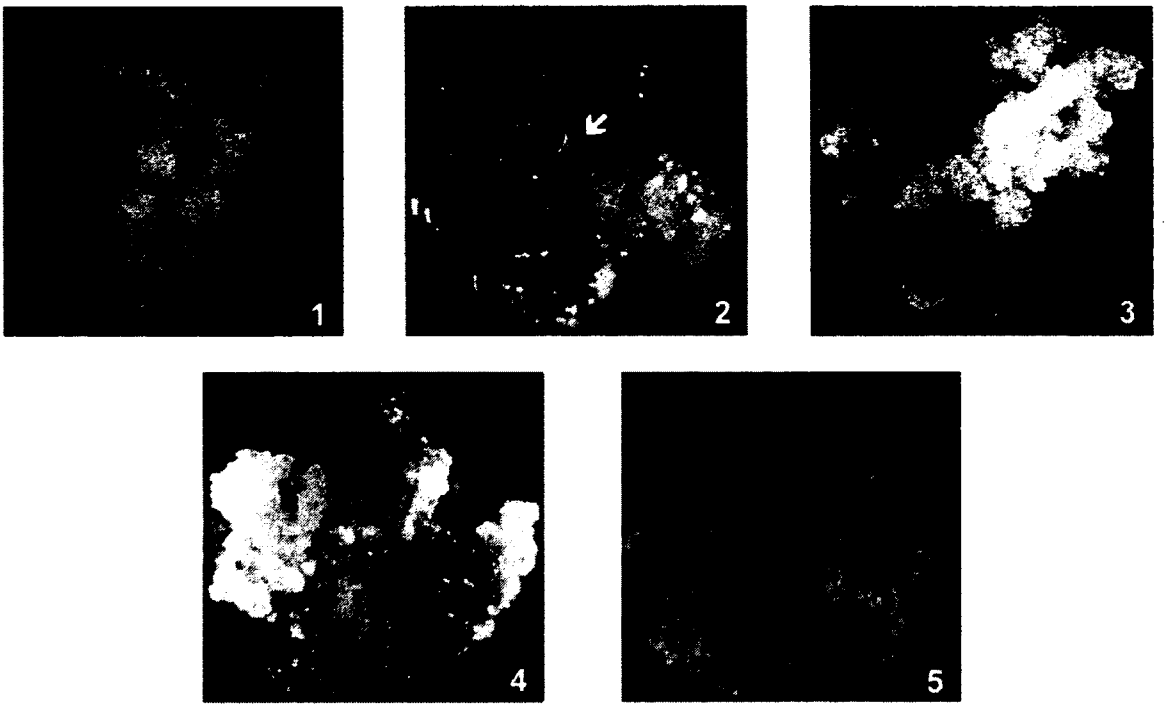


Figure 7. Callus Degeneration as Seen with Three Constructs. 1. By Week 17, the constructs *FRASUP1*-OE, *AGAMOUS*-OE, and *FRASUP3*-R calli started to turn white. 2. By Week 19, the white parts of some calli began to secrete a gel-like liquid (indicated by arrow) and turn light brown. 3. By Week 21, the gel-like liquid dried. 4. After the 21st week, these degenerating calli turned from brown to black. 5. By Week 24, degenerating calli had turned completely brown and were considered to be dead.

Table 4. Summary of Responses to Co-cultivation

Constructs	Number of Explants.	Number of Explants Forming Callus	Number of Calli Regenerating Shoots	§ Plants Regenerated	Results of PCR Test for Inserts
FRASUP1-R	150	62	61	6	All Positive
FRASUP1-OE	150	33	26	0	N/A
FRASUP2-R	150	51	47	3	All Negative
FRASUP2-OE	150	82	63	8	All Negative
FRASUP3-R	150	7	7	0	N/A
FRASUP3-OE	250	0	N/A	N/A	N/A
FRASUP4-R	150	11	10	0	All Positive
FRASUP4-OE	250	0	N/A	N/A	N/A
FRASUP5-R	150	44	34	1	All Positive
FRASUP5-OE	250	0	N/A	N/A	N/A
AGAMOUS-R	150	120	81	2	All Positive
AGAMOUS-OE	150	104	33	0	N/A
ACS-7-R	150	61	49	1	N/A
ACS-7 OE	150	118	57	28	All Positive

§ Each plant has regenerated from a different explant.

3.3 GFP Screening

Beginning at 4 weeks (week 4) after culture, GFP expression was checked in all calli, after which GFP assays of calli and regenerating shoots and plantlets were continued throughout the experiment. At week 4 some cell clusters expressing GFP were observed. Most of these GFP^+ calli had by this time already developed meristematic nodules. But the calli of the constructs *FRASUP3*-OE, *FRASUP4-OE*, and *FRASUP5*-OE died soon after callus initiation, and were not tested for GFP expression.

By week 7, the surviving leaf explants tended to form large and isolated calli with strong GFP expression, and GFP^- calli were rarely found (only one *FRASUP4* RNAi callus was GFP^- at week 7, and died soon after). When culture was continued until 8 weeks after co-cultivation, almost all calli were GFP^+, and 67% of the GFP^+ calli had formed shoots by that time. Once shoot formation had initiated, few new calli formed on the explants.

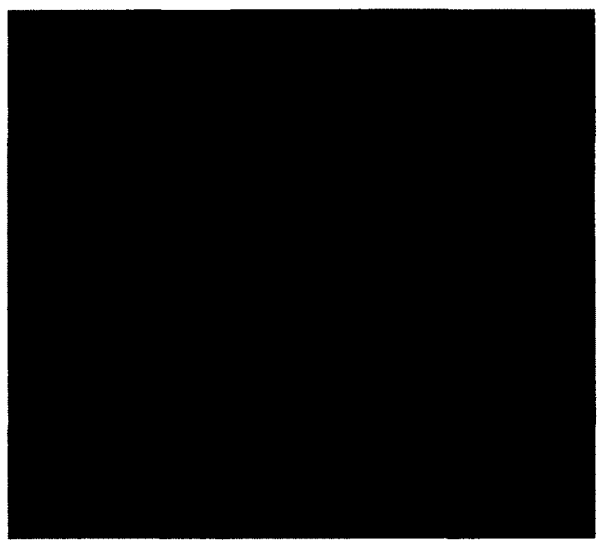


Figure 8. An Example of GFP^+ Callus. This transformed callus, approximately 7 to 8 weeks after co-cultivation, carried insert *AGAMOUS*-R.

3.4 Phenotypes of Transformed Plants

SUPERMAN Gene Transformants

Transformed plants were obtained with RNAi constructs *FRASUP1*-R, *FRASUP2*-R, *FRASUP4*-R, and *FRASUP5*-R, and overexpression construct *FRASUP2*-OE. These plants exhibited GFP fluorescence, but no other phenotypic differences from wild type could be seen. As previously noted, transformed callus carrying the overexpression constructs *FRASUP3-OE*, *FRASUP4*-OE, and *FRASUP5*-OE died at the callus stage, and therefore could not be regenerated. This lethality constitutes a kind of phenotype that is evident at the callus stage. With two other constructs, *FRASUP1*-OE, and *FRASUP3*-R, callus formed, but ultimately died without any visible evidence of differentiation, some phenotypes lacking differentiation.

AGAMOUS Gene Transformants

Among the initial 27 plant regenerants that had been transformed with the *AGAMOUS*-R construct, three independently derived plants (i.e., plants regenerated from different calli) initially had petal-like stamens but with normal pistils (Figure 9). But this phenotype was seen only immediately after these three transformants were transferred from the culture room to the greenhouse. Subsequently developing flowers had a normal phenotype. In a subsequent set of *AGAMOUS*-R regenerated plants, three more individual flower-abnormal phenotypes were found. Unlike the petal-like stamen, this phenotype had normal sepals, stamen, and carpels, and even normal fruit formation, but they had vestigial petals that had adaxial concavity (Figure 10). In contrast, transformation

with the *AGAMOUS*-OE construct produced callus that did not differentiate, and that appeared dead by 24 weeks of co-cultivation.

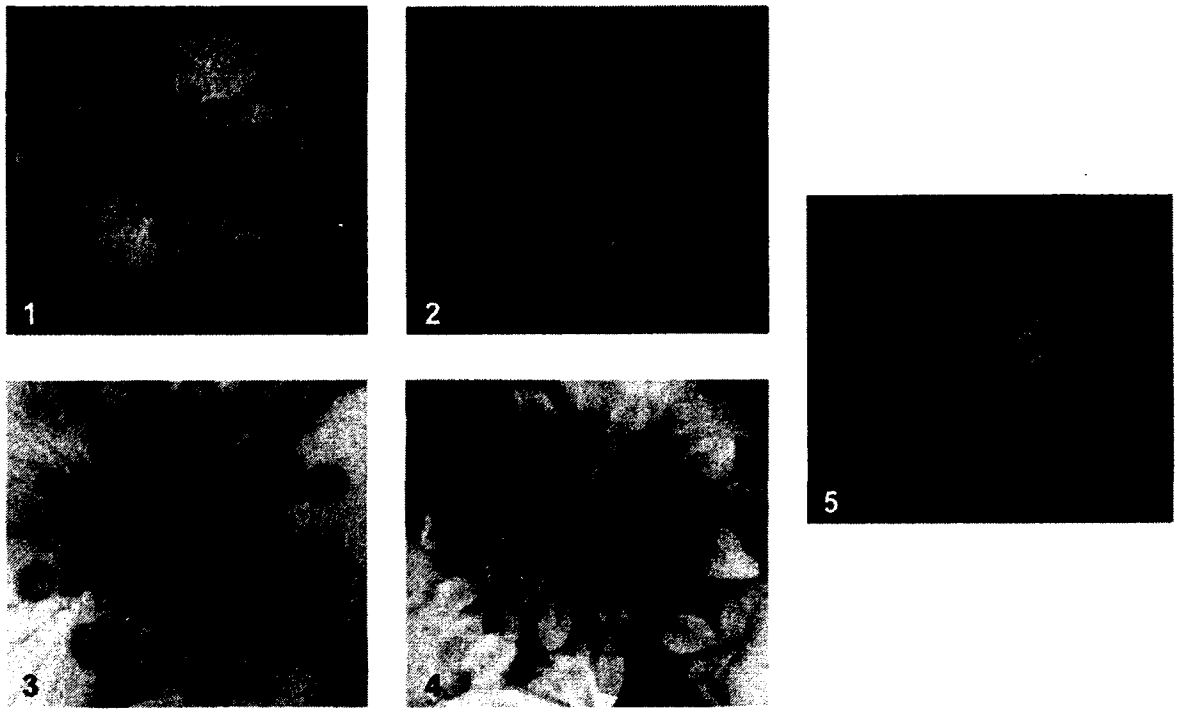


Figure 9. Phenotype of Initial *AGAMOUS* RNAi Transformants with Petalloid Stamens. **1.** Top view of wild type flower. **2.** Top view of aberrent flowers. **3.** Reproductive organs of wild type flower. **4.** Reproductive organs of aberrent flowers. **5.** Comparison between normal (wild type) stamen (Left) and petalloid stamen (Right).

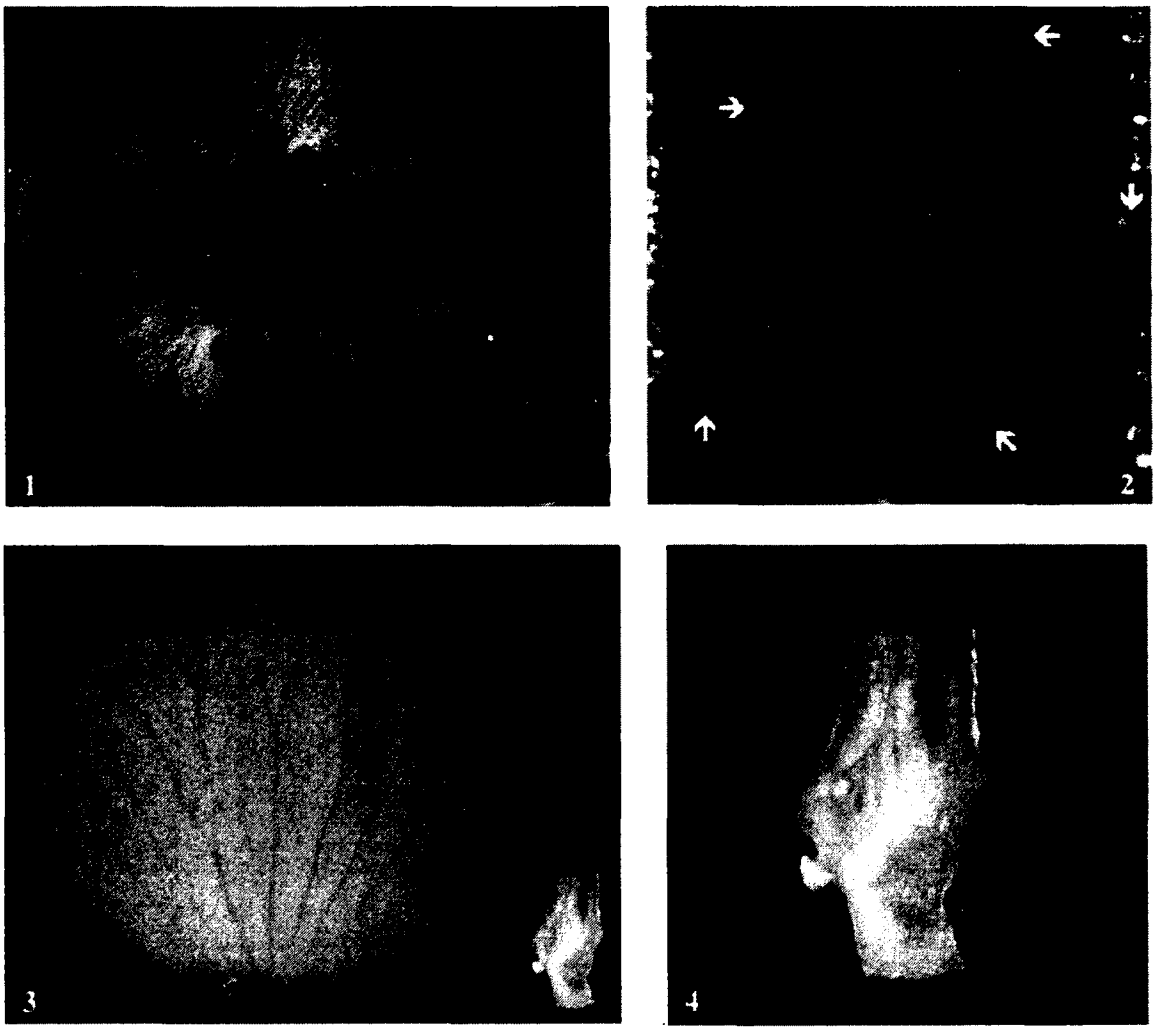


Figure 10. Phenotype of Second Set of *AGAMOUS* RNAi Transformants with Aberrant Petals. **1.** Comparative sizes of wild type flower and aberrant flowers (see inset with same magnification). **2.** Enlarged view of aberrant flower. The vestigial petals are indicated by arrows. **3.** Comparative sizes of wild type and aberrant petals. **4.** Enlarged view of an aberrant petal.

ACS-7 Gene Transformants

An aberrant phenotype was seen in 10 of 28 *ACS-7*-OE transgenic plants. These aberrant plants were smaller sized in every organ and had much slower growth. They had shrunken, distorted flowers (Figure 11), but did bear fruit. The foliage of both *ACS-7*-R and -OE transformed plants displayed the same phenotype (not shown), but the -R transformants had not yet flowered after 15 months after regeneration.

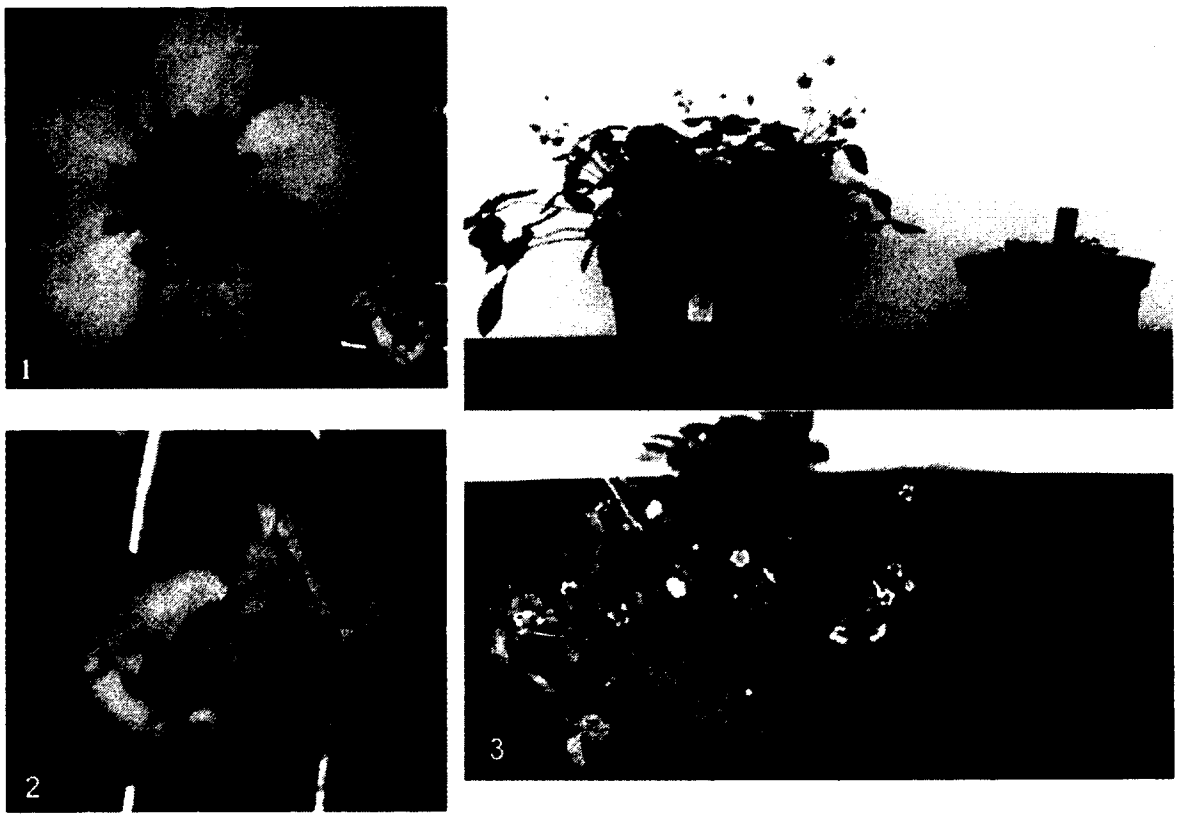


Figure 11. Phenotype of *ACS-7* Overexpression Transformants. **1.** Comparative sizes of wild type flower and aberrant OE flower (see inset with same magnification). **2.** Enlarged view of aberrant flower. **3.** Comparative sizes of wild type plant and transformant plant after 16 weeks grown in pot.

3.5 Insert Testing of Transformed Plants

Summed over all of the constructs, approximately 110 transformed plants were regenerated. However some positive transformant plantlets died. A total of 92 regenerated plants were genotyped by PCR for presence of the appropriate insert using a universal vector primer and a gene specific primer (Table 5). Out of the 92 plants genotyped, 81 of them yielded positive results giving PCR products of the appropriate sizes (Table 3). None of the 11 *FRASUP2* transformant plants (3 –R, 8 –OE) gave a positive result with the PCR test, although all were GFP positive. An example of genotyping results is shown in Figure 12.

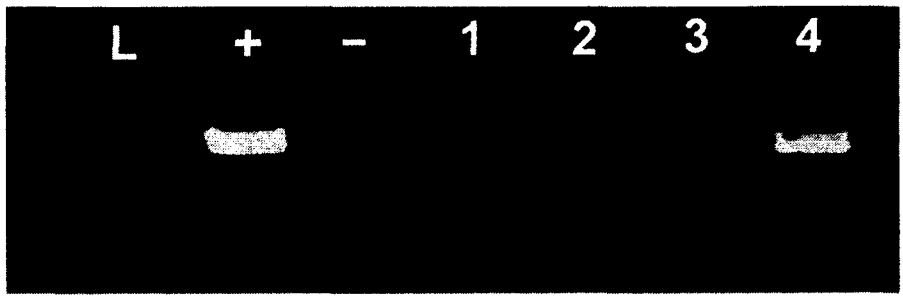


Figure 12. An Example of Insert Testing by PCR. L. Marker. + Positive control. –. Negative control (see Table 2). 1-4. Products generated by genotyping PCR of different lines of *AGAMOUS* RNAi 1-4

Table 5. PCR Primers Used for Insert Testing (* indicates primer obtained from Benjamin Orcheski, 2010).

Gene Name	Forward Primer Reverse Primer	Primer Sequences	Expected Product Size	Observed Product Size
FRASUP1	p35SF FRASUP1 R Trans	5'-ATCAAGACGATCTACCCGAGTAATAAT-3' 5'-AGAAAGCTGGGTCTAGAGATCGGCGAGTCGAA-3'	668	640
FRASUP2	p35SF *FRASUP2 R Trans	5'-ATCAAGACGATCTACCCGAGTAATAAT-3' 5'-AGAAAGCTGGGTTTAGCCTAATCGAAGCTCTA-3'	1583	N/A
FRASUP3	p35SF *FRASUP3 R Trans	5'-ATCAAGACGATCTACCCGAGTAATAAT-3' 5'-AGAAAGCTGGGTTCAGTAGCCTAATCGAAGCT-3'	1678	N/A
FRASUP4	p35SF *FRASUP4 R Trans	5'-ATCAAGACGATCTACCCGAGTAATAAT-3' 5'-AGAAAGCTGGGTTCATTTGATCTTGGGAGTTG-3'	1786	1740
FRASUP5	p35SF FRASUP5 R Trans	5'-ATCAAGACGATCTACCCGAGTAATAAT-3' 5'-AGAAAGCTGGGTTTAATCTACTTTCATTAGCA-3'	1554	1530
ASC-7	p35SF *ACS-7 R Trans	5'-ATCAAGACGATCTACCCGAGTAATAAT-3' 5'-AGAAAGCTGGGTCTAGTGCCTTCTCTCTCTTT-3'	3735	3770
AGAMOUS	p35SF AG SR	5'-ATCAAGACGATCTACCCGAGTAATAAT-3' 5'-CCCGCATCAGCGTTCAACTCTAGT-3'	1053	974

CHAPTER 4

DISCUSSIONS & CONCLUSIONS

The overall project goal was to determine whether any of seven candidate genes, re-introduced into diploid strawberry in the form of overexpression and RNAi constructs, would affect sex-determination or the development of sex organs, and thus provide insight into the genetic basis for femaleness in strawberry. The candidate genes in question were chosen for variety of reasons. According to the "ABC Development Model", the *AGAMOUS* gene is expected to serve a vital role in directing reproductive organ development of the strawberry flower. The *ASC-7* gene is expected to be involved in synthesis of ethylene in strawberry, and plays a direct role in the suppression of stamen development in *Cucumis melo* (Boualem et al., 2008). The *FRASUP1* ~ *FRASUP5* have high homology with *SUPERMAN* genes from other species, which belong to the *SUPERMAN*-like *RBE-SUP* sub-family. These genes all have been studied previously in other organisms, such as *Arabidopsis* (Shultz, 1991; Bowman, 1992; Sakai, 1995;Yun et al, 2002) and tobacco (Bereterbide et al. 2002), whereas, until now, in strawberry no previous work has been done.

As described below, several of the recombinant constructs produced interesting phenotypes, some including effects on floral development, and others affecting the capacity to survive and regenerate from callus culture. Despite the success with transformation, the outcome with six of the 14 constructs was an early or eventual death of the initiated callus, thus precluding regeneration of

plants and examination of effects on transgene retention and floral traits. Each abnormal phenotype was seen in at least two or three independent transformants. Thus, the observed phenotypes were probably not due to the effects of insertional mutations in endogenous genes.

Three of the overexpression constructs, *FRASUP3-OE*, *FRASUP4-OE*, and *FRASUP5-OE*, could not survive beyond callus initiation. This outcome was unlikely to have been due to procedural problems, because: 1) co-cultivation was repeated with these constructs five times with 50 explants each, always with the same results; and 2) regeneration was achieved with several other constructs, indicating that the regeneration procedure was appropriate and effective. Also, these outcomes are highly unlikely to be due to insertional mutation, since many independent transformations were obtained and they yielded the same outcomes. Thus, the rapid death of these newly initiated calli was most probably caused by the introduced transgenes, the products of which triggered or blocked some vital cellular process or processes, resulting in rapid death.

Three other constructs, *FRASUP1*-OE, *AGAMOUS*-OE, and *FRASUP3*-R, initiated calli that grew for several weeks, but failed to differentiate and ultimately died. There are two likely explanations for this result. The failure of the calli to differentiate with these last three constructs might have been simply because the calli were dying, although more slowly than with the first three constructs. Thus, it might not have been necessarily an inability to differentiate, but simply a different (slower) path to lethality. The second explanation is that extra *FRASUP1* and *AGAMOUS* gene products or the knocking-out of the *FRASUP3* gene may impair

the plant cells' ability to function properly, especially during differentiation. By considering the fact that both overexpression and knocking-out of the *FRASUP3* gene caused callus death, we might conclude that the *FRASUP3* gene plays a vital role in *F. vesca* gene expression, metabolism, cell division, or other vital processes. Further study of these genes would benefit from the availability of an inducible or flower-specific promoter, so that expression of the transgene could be delayed until after regeneration, providing opportunity to see whether regeneration could be obtained and whether any floral or other phenotype would subsequently be seen *in vivo*.

Transformed plants were obtained with both overexpression and RNAi constructs of the *FRASUP2*; however, when PCR tests were performed none of these regenerants yielded positive evidence of the present of the introduced gene constructs. The 11 transformant plants (3-R and 8-OE) that survived selection were GFP^+, but none yielded a PCR product for presence of the *FRASUP2* gene. The reason might be that the construct lost the *p35S* promoter or other portion of the *FRASUP2* sequence to which the PCR primers were targeted, but still contained intact GFP sequence and the selective (antibiotic resistance) marker gene. One possible reason might be that the left and right borders of the insert sequence tend to be lost when transferring into plant genomes, so *FRASUP2* constructs may lose one of the primer sites but still contain GFP site. But this does not explain why such loss occurred only with one insert, in both RNAi and OE constructs. Thus, perhaps the *FRASUP2* insert itself has an effect on construct stability.

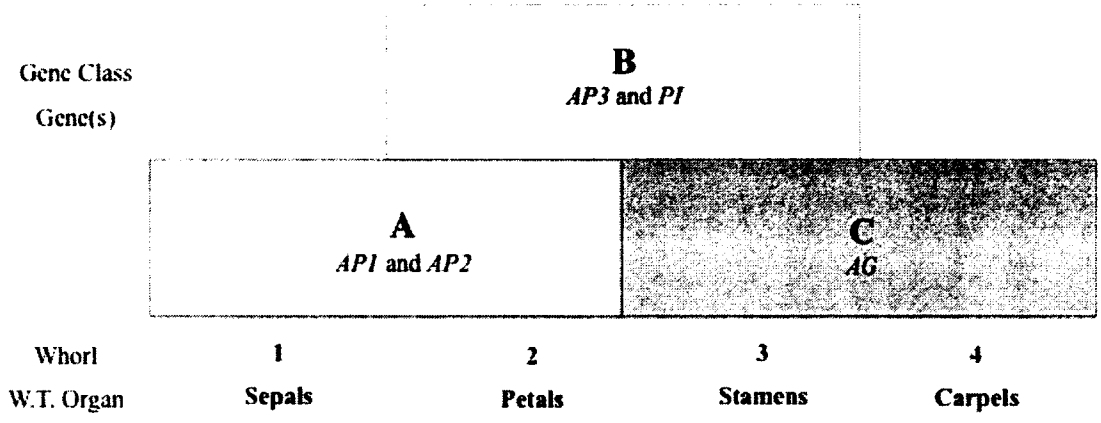
Considering the fact that femaleness in strawberry is, in the classical model (Ahmadi and Bringhurst, 1991) determined by a dominant allele, the *SUPERMAN* family genes make excellent candidates because the gain of function *SUP* mutations, ectopic and overexpression, might mimic the phenotype of female *F. virginiana* flowers: First, femaleness in strawberry is manifested by the presence of vestigial stamens (Figure 4). Many studies have demonstrated that ectopic or overexpression of *SUP* could severely reduce the size of stamens (Kater et al., 2000; Yun et al., 2002; Bereterbide et al., 2001). Second, the number and position of the vestigial stamens do not change in female flowers. Bereterbide et al. (2001) reported that under some circumstances overexpression of *SUP* mutants displayed this feature. Finally, the female flowers of strawberry are much smaller than hermaphrodites. The phenotype of smaller flowers is also observed in petunia flowers that overexpress the *PhSUP1* gene. Flowers on these plants have an average reduction in size of 60-70% (Nakagawa et al., 2004).

Introduction of the *AGAMOUS* RNAi construct caused two distinct floral phenotypes in *F. vesca*. The phenotype containing petal-like stamens (Figure 9) partially confirmed our expectation that whorl 3 and whorl 4 might be converted into petals, or petal-like structures, due to knocking out of the *AGAMOUS* gene, because according to the "ABC Development Model", *AGAMOUS* is known to serve as a negative regulator to both A- and B-class genes (Figure 13). The incomplete realization of these expectations that we observed might be due to insufficient expression of the RNAi construct. But the result still suggests that the

AGAMOUS gene indeed serves as a negative regulator to A- and B-class genes in strawberry.

However, the previously described phenotypes were only seen in a few *AGAMOUS* regenerants immediately after these transformants were transferred from the culture room to the greenhouse. Subsequently developed flowers had a normal phenotype. Thus, the temporary abnormal floral morphology might have been triggered by stresses associated with in vitro culture and regeneration. But since only three of 27 lines had this phenotype, with the remainder displaying a wild type phenotype, it is less likely that a stressful environment alone is the cause. Instead, perhaps the effect of the transgene insertion is subtle, but is magnified in an unknown way by physiological effects of the in vitro culture and regeneration process. This explanation is consistent with the temporary nature of the phenotype. This phenotype is an interesting discovery and needs further study to reach a conclusion about the effects of *AGAMOUS* gene manipulation on strawberry flowers.

A.



B.

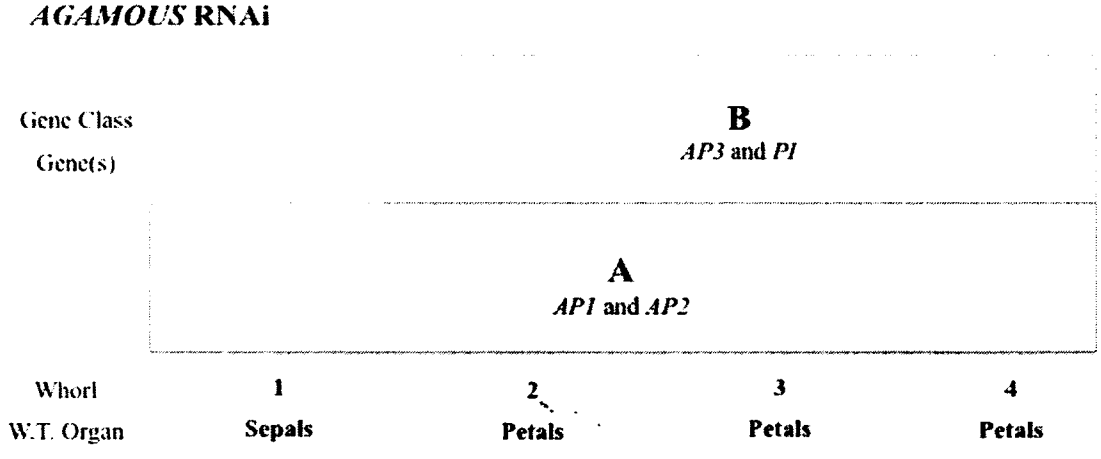


Figure 13. Comparison of General Model and Predicted Model for the Phenotypic Consequences of *AGAMOUS* RNAi Construct. A. According to the general "ABC Development Model", the C-class *AGAMOUS* gene expresses in whorls 3 and 4 (Meyerowitz et al. 1991). **B.** When the C-class *AGAMOUS* gene is silenced, its expression and influence would be lacking in whorls 3 and 4, allowing the A-class gene expression to extend to whorls 3 and 4, and B-class gene expression to extend to whorl 4 (Meyerowitz et al. 1991). This might result in petal development in whorl 3 and even into whorl 4, producing petal-like stamens and carpels, or undeveloped stamens and carpels. Our results in strawberry, namely petal-like stamens, are consistent with this model.

Another phenotype seen in some *AGAMOUS* RNAi regenerants, vestigial petals (Figure 10), is difficult to explain on the basis of the ABC model. This transformed phenotype has functional whorls 1, 3, and 4, but the petals in whorl 2 are vestigial. Therefore, the vestigial petals are unable to serve their normal functions, such as protection of the sexual organs in the flower bud, or the attraction of pollinators. The observed phenomenon of vestigiality in one whorl, in this case whorl 2, is similar in this respect to femaleness (vestigial whorl 3) and maleness (vestigial whorl 4). However, since presence/absence of petals is not associated with sex type in strawberry, this phenotype, i.e. vestigial petals, may be not very significant to this research.

In *Arabidopsis*, the *AGAMOUS* gene is able to regulate floral meristem determinacy, but when there was a loss of function, it was found that multiple whorls and flowers develop inside the fourth whorl (Meyerowitz et al. 1991). It followed that the overexpression of the *AGAMOUS* gene could inhibit the development of meristems, which seems a likely explanation for the non-differentiation-of-callus phenotype we observed. In sum, seeing the two transformed phenotypes, i.e. petal-like stamens and vestigial petals, promisingly leads us to believe that the *AGAMOUS* gene can affect the development of strawberry flower, and perhaps contribute to sex determination, even though one phenotype is unstable (petal-like stamens) and another does not fit the ABC model (vestigial petals).

The *ACS* gene family controls the synthesis rate of ethylene, which serves as a vital gaseous hormone and is involved in many regulatory processes in

plants. In response to introduction of the *ACS-7*-R construct, which was intended to knock out expression of this gene, we saw smaller organ size and much slower growth. These results could be due to smaller cells or fewer cells. To determine which is the case, we could look at the comparative sizes of leaf epidermal cells, which could be done with leaf peels wet-mounted on microscope slides. In contrast, when the *ACS-7*-OE construct was introduced, with the intention of overexpressing this gene, the transgenic plants also had smaller size organs and much slower growth (Figure 11). However, no ACS-7-OE transformant flowered, so we could not know whether any phenotype would be shown. Once the *ACS-7* gene was overexpressed or knocked out, we observed that the transgenic plants all had smaller organs and slower growth process, with the RNAi transformed plants also having abnormal flowers. Thus, we believe that the insufficiency or surplus of *ACS-7* gene products inhibited the ethylene synthesis and then in turn impaired the associated regulatory processes. In *Cucumis melo*, the activity of *CmACS-7* significantly affected the development of stamens (Boualem et al., 2008), but did not seem to suppress the growth of the whole plant. However, overexpression of inserted gene would also lead to the same phenomenon of RANi (Hagiwara-Komoda et al., 2008). Hence, our further study would quantify the expression amount of inserted constructs, and focus on the relationship between the amount of exogenous ethylene and the development of stamens in strawberry.

STUDY SUMMARY

This study contributed toward identifying the genetic cause of sex determination in octoploid *Fragaria virginiana* by examining the effects of over-expression and RNAi constructs of seven candidate genes when introduced into the hermaphroditic, diploid strawberry, *Fragaria vesca* 'Hawaii-4'. In total, 14 constructs of seven genes, including five members of the strawberry *SUPERMAN*-like gene family, were transferred into strawberry, yielding some fascinating phenotypes. Although none of these introduced constructs produced phenotypes that mimic the vestigial stamen phenotype of female *F. virginiana* flowers, six constructs had some kind of effect on flowering or floral morphology. However, since another six transformant types were not regenerable and another never flowered, there may still be an opportunity to observe effects on floral phenotype among these constructs if regenerated plants can be obtained and maintained until flowering.

FUTURE PLANS

Although we observed some intriguing phenotypes and indeed answered some questions about the effects of manipulating seven candidate genes, there is still much research needed in order to find an answer regarding sex determination in strawberry. As a continuation to this research, one short-term goal is to collect the mature seeds from T1 generation (the transformed plants) and germinate them to observe the phenotype(s) of T2 generation (the progeny of transformants). Those T2 lines are invaluable for several reasons. First, T2 lines might show additional phenotypes or ones that differ from those observed in T1. This could be because homozygous T2 plants increase the transgene copy number or because some silenced transgenes become un-silenced in T2 generation. Second, we could see the inherited stability of T-DNA inserts in subsequent generations. Third, the T2 generation would clarify whether those phenotypes seen in T1 can be attributed to transformation or were caused by activating an endogenous gene through the 35S promoter in T-DNA or were knockouts that were the result of T-DNA inserting into a native gene. Typically, the phenotypic effects of insertional knockouts are not seen until the T2 generation, because the recessive knockout allele needs to be homozygous to produce a phenotype.

Another short-term goal is to develop further constructs of these candidate genes with an inducible promoter or flower-specific promoter. This would provide opportunity to see whether candidate genes used in non-

regenerable constructs in the present study would have floral phenotypes when expressed only after the regeneration phase.

In addition, in this research, the construct *AGAMOUS* RNAi caused two discrete phenotypes. Since different insertion sites could lead to dramatic variations of phenotype, even with a same construct, it would be helpful to identify the insertion sites for each construct. Among the methods available for this identification, one possibility is to use SiteFinding PCR (Tan G. et al, 2005), or another could be global mapping (Gabriel A. et al, 2006).

Although many questions remain to be answered, this project generated new knowledge, and interesting transformant plants that can provide a foundation for continued investigations.

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